Electronic Supplementary Material (ESI) for Chemical Science.
This journal is © The Royal Society of Chemistry 2017

Supporting Information

Synthesis and Stabilities of Peptide-based [1]Rotaxanes: Molecular Grafting onto Lasso Peptide Scaffolds

*Fumito Saito[1] and Jeffrey W. Bode[1]**

[1]Laboratorium für Organische Chemie, Department of Chemistry and Applied Biosciences,

ETH-Zürich, 8093 Zürich, Switzerland

bode@org.chem.ethz.ch

Table of Contents

1. General Methods

1.1. Reactions and purifications

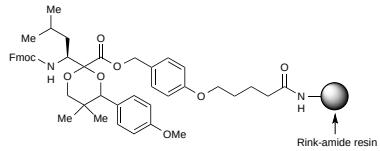
Reactions were carried out under air otherwise stated. Thin layer chromatography (TLC) was performed on Merck TLC plates (0.25 mm) pre-coated with silica gel 60 F254 and visualized by UV quenching and/or staining with ninhydrin solution and warming with a heat gun. Flash column chromatography was performed under a forced-flow of air using Silicycle SiliaFlash F60 (40-63 mm particle size). Peptides were analyzed and purified by reversed phase high performance liquid chromatography (RP-HPLC) on Jasco analytical and preparative instruments with dual pumps, mixer and in-line degasser, a variable wavelength UV detector (simultaneous monitoring of the eluent at 220 nm, 254 nm, 301 nm) and a Rheodyne 7725i injector fitted with a 20 to 1000 μL injection loop. The mobile phase for analytical and preparative HPLC were Millipore-H_2O with 0.1% TFA (Buffer A) and HPLC grade CH_3CN with 0.1% TFA (Buffer B). Analytical HPLC was performed on Shiseido C18 (5 μm, 4.6 mm I.D. x 250 mm) column at a flow rate of 1 mL/min. Analytical HPLC traces (λ = 220 nm) used for reaction monitoring were obtained with the following method: 20 to 95% CH_3CN with 0.1% TFA in 17 min, then 95% CH_3CN with 0.1% TFA for 7 min. Preparative HPLC was performed on YMC C18 (5 μm, 20 mm I.D. x 250 mm) column at a flow rate of 10 mL/min. LCMS analysis was performed on Dionex UltiMate 3000 RSLC connected to a Surveyor MSQ Plus mass spectrometer; a reversed-phase RESTEK Pinnacle II C18 (4.6 x 50 mm) column was used, running a gradient of 5 to 100% CH_3CN in H_2O over 4.5 min, 100% CH_3CN for 2.5 min.

1.2. Characterization

NMR spectra were recorded on a Bruker AV-400, AV-III-600. Chemical shifts (δ) are given in ppm relative to residual solvent peaks. Data for ^1H NMR are reported as follows: chemical shift (multiplicity, coupling constants where applicable, number of hydrogens). Abbreviations are as follows: s (singlet), d (doublet), t (triplet), q (quartet), p (pentet), dd (doublet of doublet), m (multiplet), br (broad). In ^{19}F and ^{11}B NMR, multiplets are reported as the average of the observed signals. IR spectra were recorded on a Jasco FT/IR-4100 spectrometer and only major peaks are reported in frequency of absorption (cm^{-1}). Optical rotations were measured on a Jasco P-2000 operating at the sodium D line with a 100 mm path length cell. High-resolution mass spectra were obtained by the mass spectrometry service of the ETH Zürich Laboratorium für Organische Chemie on a Bruker Daltonics maXis ESI-QTOF spectrometer (ESI), or a Bruker Daltonics solariX spectrometer (MALDI). Analytical HPLC traces (λ = 220 nm) used to confirm the purity of peptides were obtained with the following method: 20 to 95% CH_3CN with 0.1% TFA in 17 min, then 95% CH_3CN with 0.1% TFA for 7 min.

1.3. Solvents and reagents

All organic solvents (acetone, CH_3CN, $CHCl_3$, CH_2Cl_2, DMF, DMSO, Et_2O, MeOH) were used as supplied (ACS or HPLC grade) otherwise stated. THF was purified by distillation from sodium benzophenone ketyl prior to use. H_2O used for reactions was obtained from Millipore purification system. Potassium acyltrifluoroborates[1] and protected leucine α-ketoacid resin **S1**[2] were prepared by reported procedures. All other starting materials were used as supplied by commercial vendors or prepared by the method described in the corresponding reference.



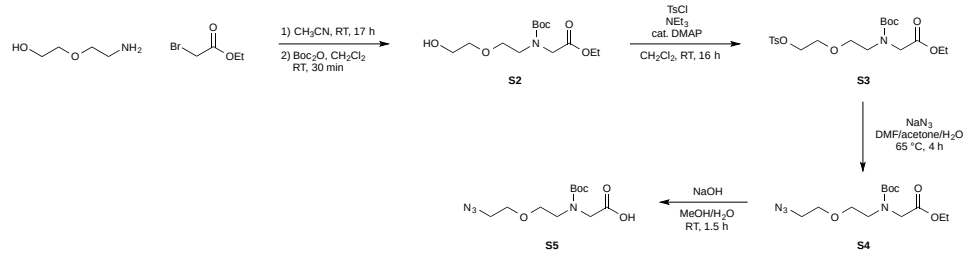
S1

1.4. Solid phase peptide synthesis

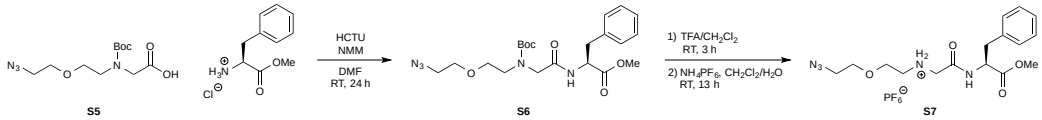
The following Fmoc amino acids and Boc amino acid with side-chain protecting groups were used: Fmoc-Ala-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Gly-OH, Fmoc-His(1-Trt)-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Nle-OH, Fmoc-Phe-OH, Fmoc-Ser(tBu)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Trp(Boc)-OH, Fmoc-D-Trp(Boc)-OH, Fmoc-Tyr(tBu)-OH, Boc-Cys(StBu)-OH. SPPS was performed on rink-amide polystyrene resin. Fmoc deprotections were performed with 20% piperidine in DMF (7 min x 2). Couplings were performed with Fmoc (or Boc) amino acid (4.0 equiv to resin substitution), HCTU (4.0 equiv) and NMM (8.0 equiv) in DMF for 2 h.

2. A feasibility study for the formation of a [2]rotaxane with the KAT-azide coupling reaction

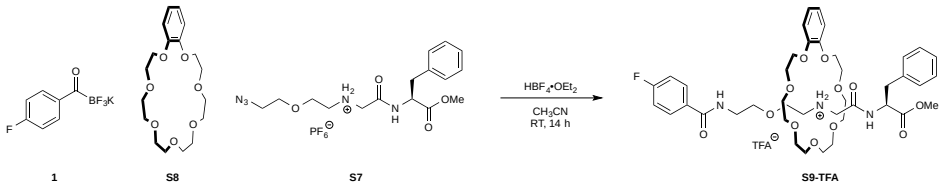
a)



b)

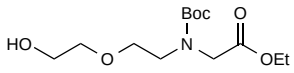


c)



Scheme S1. Initial feasibility study for the formation of [2]rotaxane **S9-TFA**.

Alcohol S2



S2

To a solution of 2-(2-aminoethoxy)ethanol (27.2 mL, 270 mmol, 3.0 equiv) in CH_3CN (120 mL) was added ethyl bromoacetate (10.0 mL, 90.0 mmol, 1.0 equiv). The mixture was stirred at RT for 17 h and CH_3CN was evaporated under reduced pressure. The residue was dissolved in CH_2Cl_2 and washed with H_2O (3x). The combined aqueous phases were back-extracted with CH_2Cl_2 (1x). The combined organic phases were dried over Na_2SO_4, filtered and concentrated under reduced pressure.

The residual oil was dissolved in CH_2Cl_2 (250 mL) and Boc_2O (19.6 g, 90 mmol, 1.0 equiv to ethyl bromoacetate) was added to this solution. The mixture was stirred at RT for 30 min and CH_2Cl_2 was evaporated under reduced pressure. The residue was purified by flash column chromatography (hexanes/EtOAc 1:2) to give **S2** (4:6 rotamers by ^{1}H NMR integration, 5.8 g, 34% yield over 2 steps) as a colorless oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ 4.19-4.13 (m, 2H), 4.01 (s, 2H x 0.4), 3.94 (s, 2H x 0.6), 3.68-3.64 (m, 2H), 3.61-3.56 (m, 2H), 3.52-3.42 (m, 4H), 2.41 (br s, 1H), 1.44-1.40 (m, 9H), 1.27-1.22 (m, 3H).

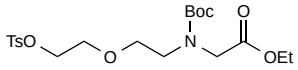
^{13}C NMR (100 MHz, $CDCl_3$) δ 170.50, 170.31, 155.41, 155.25, 80.30, 72.32, 70.24, 70.16, 61.62,

61.58, 60.96, 50.66, 50.03, 48.47, 48.20, 28.30, 28.15, 27.35, 14.19, 14.08.

IR (thin film) 3480, 2978, 2935, 2873, 1752, 1701, 1458, 1401, 1367, 1250, 1198, 1172, 1144 cm^{-1}.

HRMS (ESI) calcd for $C_{13}H_{25}NNaO_6$ $[M+Na]^+$: 314.1574, found: 314.1575.

Tosylate S3



S3

To a solution of **S2** (2.0 g, 6.9 mmol, 1.0 equiv) in CH_2Cl_2 were added NEt_3 (1.15 mL, 8.3 mmol, 1.2 equiv), DMAP (8.4 mg, 69 μmol, 0.01 equiv) followed by TsCl (1.6 g, 8.3 mmol, 1.2 equiv). The mixture was stirred at RT for 16 h and CH_2Cl_2 was evaporated under reduced pressure. The residue was purified by flash column chromatography (hexanes/EtOAc 3:1 to 2:1) to give **S3** (4:6 rotamers by ^1H NMR integration, 2.6 g, 84% yield) as a colorless oil.

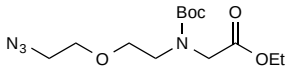
^1H NMR (400 MHz, $CDCl_3$) δ 7.80-7.78 (m, 2H), 7.34 (d, *J* = 8.0 Hz, 2H), 4.19-4.14 (m, 2H), 4.13-4.09 (m, 2H), 3.95 (s, 2H x 0.4), 3.90 (s, 2H x 0.6), 3.61-3.56 (m, 2H), 3.54-3.50 (m, 2H), 3.42-3.35 (m, 2H), 2.44 (s, 3H), 1.44-1.41 (m, 9H), 1.29-1.24 (m, 3H).

^{13}C NMR (100 MHz, $CDCl_3$) δ 170.22, 170.09, 155.40, 155.20, 144.85, 144.80, 133.00, 132.93, 129.82, 129.80, 127.91, 80.31, 80.23, 70.67, 70.62, 69.02, 68.47, 68.32, 60.88, 60.83, 50.59, 49.85, 48.15, 47.87, 28.32, 28.18, 21.61, 14.27, 14.16.

IR (thin film) 2978, 1750, 1699, 1365, 1190, 1177, 1142 cm^{-1}.

HRMS (ESI) calcd for $C_{20}H_{32}NO_8S$ $[M+H]^+$: 446.1843, found: 446.1839.

Azide-ethyl ester S4



S4

To a solution of **S3** (3.7 g, 8.3 mmol, 1.0 equiv) in DMF/acetone/H_2O (36 mL, 1:1:1) was added NaN_3 (0.81 g, 12 mmol, 1.5 equiv). The mixture was stirred at 65 °C for 4 h and cooled to RT. The mixture was diluted with CH_2Cl_2 and washed with H_2O (3x), then brine (1x). The organic phase was dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was purified by flash column chromatography (hexanes/EtOAc 3:1) to give **S4** (4:6 rotamers by ^1H NMR integration, 2.3 g, 88% yield) as a pale yellow oil.

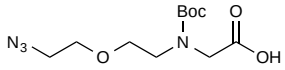
^1H NMR (400 MHz, $CDCl_3$) δ 4.21-4.14 (m, 2H), 4.05 (s, 2H x 0.4), 3.98 (s, 2H x 0.6), 3.64-3.58 (m, 4H), 3.52-3.44 (m, 2H), 3.35-3.31 (m, 2H), 1.46-1.42 (m, 9H), 1.29-1.24 (m, 3H).

^{13}C NMR (100 MHz, $CDCl_3$) δ 170.29, 170.15, 155.45, 155.24, 80.31, 80.23, 70.65, 70.52, 69.90, 69.79, 60.89, 60.82, 50.74, 50.71, 49.95, 48.37, 48.14, 28.35, 28.20, 14.27, 14.15.

IR (thin film) 2979, 2110, 1751, 1701, 1249, 1197, 1171, 1142 cm^{-1}.

HRMS (ESI) calcd for $C_{13}H_{24}N_4NaO_5$ $[M+Na]^+$: 339.1639, found: 339.1635.

Azide-acid S5



S5

To a solution of **S4** (2.3 g, 7.3 mmol, 1.0 equiv) in MeOH (22 mL) was added 1 M aq NaOH (11 mL, 11 mmol, 1.5 equiv). The mixture was stirred at RT for 1.5 h and diluted with CH_2Cl_2 and H_2O. The aqueous phase was acidified with 1 M aq HCl to pH 2 and extracted with CH_2Cl_2 (3x). The combined organic phases were dried over Na_2SO_4, filtered and concentrated under reduced pressure to give **S5** (4:6 rotamers by ^{1}H NMR integration, 1.7 g, 81% yield) as a colorless oil.

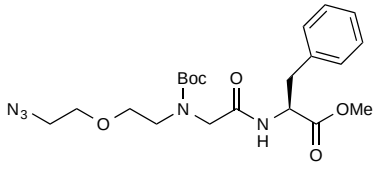
^{1}H NMR (400 MHz, $CDCl_3$) δ 9.83 (br s, 1H), 4.09 (s, 2H x 0.4), 4.04 (s, 2H x 0.6), 3.65-3.58 (m, 4H), 3.53-3.46 (m, 2H), 3.36-3.33 (m, 2H), 1.46-1.42 (m, 9H).

^{13}C NMR (100 MHz, $CDCl_3$) δ 175.60, 175.25, 155.68, 155.12, 80.83, 80.78, 70.57, 70.45, 69.91, 69.80, 50.71, 50.66, 50.63, 50.03, 48.55, 48.29, 28.30, 28.14.

IR (thin film) 2979, 2934, 2111, 1729, 1699, 1251, 1170, 1146, 1127 cm^{-1}.

HRMS (ESI) calcd for $C_{11}H_{20}N_4NaO_5$ $[M+Na]^+$: 311.1326, found: 311.1327.

Azide-Phe methyl ester S6



S6

To a solution of **S5** (1.0 g, 3.5 mmol, 1.0 equiv) in DMF (6.0 mL) were added HCTU (1.6 g, 3.9 mmol, 1.1 equiv), NMM (1.2 mL, 11 mmol, 3.0 equiv) followed by L-phenylalanine methyl ester hydrochloride (0.69 g, 3.9 mmol, 1.1 equiv). The mixture was stirred at RT for 24 h and diluted with CH_2Cl_2. The organic solution was washed with H_2O/brine (1:1, 3x), dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was purified by flash column chromatography (hexanes/EtOAc 1:1) to give **S6** (1.0 g, 64% yield) as a yellow oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.30-7.20 (m, 3H), 7.13-7.10 (m, 2H), 6.76 (d, *J* = 7.9 Hz, 1H), 4.90-4.88 (m, 1H), 3.95-3.88 (m, 2H), 3.70 (s, 3H), 3.61-3.48 (m, 4H), 3.43 (m, 2H), 3.34-3.26 (m, 2H), 3.16-3.06 (m, 2H), 1.46-1.40 (m, 9H).

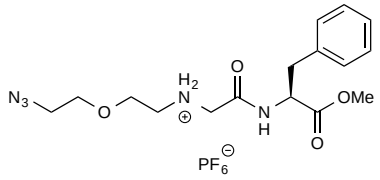
^{13}C NMR (100 MHz, $CDCl_3$) δ 171.72, 169.49, 155.11, 135.90, 129.17, 128.57, 127.06, 80.94, 69.43, 69.16, 52.96, 52.20, 50.53, 48.39, 48.11, 37.95, 28.18.

$[\alpha]_D^{25}$ = +19.3° (c 3.4, $CHCl_3$)

IR (thin film) 2976, 2932, 2869, 2108, 1746, 1699, 1525, 1456, 1366, 1248, 1173, 1144 cm^{-1}.

HRMS (ESI) calcd for $C_{21}H_{32}N_5O_6$ $[M+H]^+$: 450.2347, found: 450.2347.

Azide-Phe PF_6 salt S7



S7

To a solution of **S6** (1.0 g, 2.2 mmol, 1.0 equiv) in CH_2Cl_2 (4.0 mL) was added TFA (2.0 mL). The mixture was stirred at RT for 3 h and basified with sat aq Na_2CO_3. The aqueous phase was extracted with CH_2Cl_2 (3x). The combined organic phases were dried over Na_2SO_4, filtered and concentrated under reduced pressure.

The residue was redissolved in TFA (3.0 mL), and TFA was co-evaporated with CH_2Cl_2 (5x) under reduced pressure. To the resulting oil were added CH_2Cl_2/H_2O (30 mL, 2:1) and NH_4PF_6 (1.8 g, 11 mmol, 5.0 equiv). The biphasic mixture was vigorously stirred at RT for 13 h and two phases were separated. The aqueous phase was extracted with CH_2Cl_2 (3x). The combined organic phases were dried over Na_2SO_4, filtered and concentrated under reduced pressure to give **S7** (0.40 g, 37% yield over 2 steps) as a pale yellow amorphous.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.42 (br s, 2H), 7.29-7.25 (m, 2H), 7.22-7.13 (m, 4H), 4.83-4.76 (m, 1H), 3.98 (d, *J* = 16.1 Hz, 1H), 3.86 (d, *J* = 16.1 Hz, 1H), 3.72 (t, *J* = 5.1 Hz, 2H), 3.67 (s, 3H), 3.61 (t, *J* = 4.9 Hz, 2H), 3.38-3.36 (m, 2H), 3.30-3.18 (m, 2H), 3.13 (dd, *J* = 14.2, 5.7 Hz, 1H), 2.97 (dd, *J* = 13.8, 7.7 Hz, 1H).

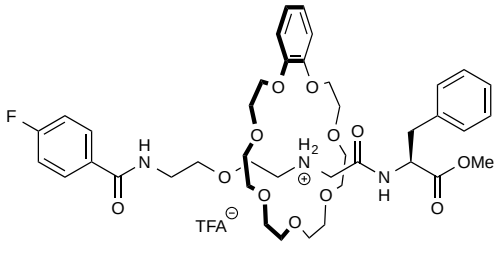
^{13}C NMR (100 MHz, $CDCl_3$) δ 171.67, 164.65, 135.63, 129.26, 128.66, 127.18, 69.78, 65.18, 54.27, 52.73, 50.43, 48.48, 48.35, 37.60.

$[\alpha]_D^{25}$ = +18.7° (c 1.0, $CHCl_3$)

IR (thin film) 3032, 2957, 2110, 1739, 1677, 1550, 1441, 1128, 842 cm^{-1}.

HRMS (ESI) calcd for $C_{16}H_{24}N_5O_4$ $[M+H-PF_6]^+$: 350.1823, found: 350.1819.

[2]Rotaxane S9-TFA



S9-TFA

4-Fluorophenyl KAT **1**[3] (84 mg, 0.36 mmol, 1.0 equiv), B21C7 **S8**[4] (0.13 g, 0.36 mmol, 1.0 equiv), and **S7** (0.18 g, 0.36 mmol, 1.0 equiv) were dissolved in CH_3CN (1.8 mL), and HBF_4•OEt_2 (98 μL, 0.72 mmol, 2.0 equiv) was added. The mixture was stirred at RT for 14 h. The reaction was quenched with H_2O. The crude material was purified by preparative HPLC using a YMC C18

column with a gradient of 20 to 95% CH_3CN with 0.1% TFA in 28 min. The pure product fractions were pooled and lyophilized to obtain 61 mg of **S9-TFA** (18% yield).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.96 (br m, 2H), 7.86-7.81 (m, 2H), 7.49 (t, *J* = 5.5 Hz, 1H), 7.24-7.15 (m, 3H), 7.10 (dd, *J* = 7.5, 1.8 Hz, 3H), 7.01 (t, *J* = 8.6 Hz, 2H), 6.91-6.87 (m, 2H), 6.83-6.79 (m, 2H), 4.76 (m, 1H), 4.40-4.21 (m, 2H), 4.18-4.13 (m, 2H), 4.07-4.04 (m, 2H), 3.88-3.83 (m, 2H), 3.68 (s, 3H), 3.67-3.39 (m, 26H), 3.15 (dd, *J* = 14.1, 5.4 Hz, 1H), 2.95 (dd, *J* = 14.1, 8.4 Hz, 1H).

^{13}C NMR (100 MHz, $CDCl_3$) δ 171.41, 166.83, 165.86, 164.55 (d, *J* = 252 Hz), 160.20 (q, *J* = 37.0 Hz),146.74, 146.72, 136.25, 130.37 (d, *J* = 2.9 Hz), 129.51 (d, *J* = 9.0 Hz), 129.08, 128.42, 126.83, 121.56, 121.53, 116.01 (q, *J* = 289 Hz), 115.23 (d, *J* = 22.5 Hz), 111.65, 111.63, 71.21, 70.74, 70.72, 70.68, 70.61, 70.55, 69.48, 69.16, 68.08, 67.98, 65.75, 53.24, 52.33, 48.39, 47.05, 39.20, 37.22.

^{19}F NMR (376 MHz, $CDCl_3$) δ –108.71.

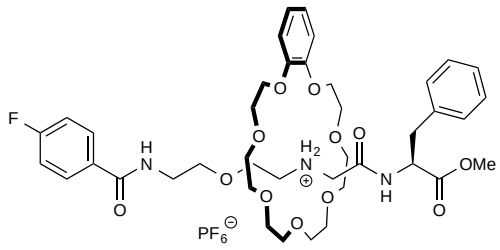
$[\alpha]_D^{26}$ = +3.7° (c 1.6, $CHCl_3$)

IR (thin film) 3068, 2905, 1742, 1691, 1504, 1250, 1200, 1118, 1104 cm^{-1}.

HRMS (MALDI) calcd for $C_{41}H_{57}FN_3O_{12}$ $[M+H–TFA]^+$: 802.3921, found: 802.3917.

To obtain a PF_6 salt **S9-PF$_6$**, **S9-TFA** was dissolved in CH_2Cl_2/H_2O (6.0 mL, 2:1) and NH_4PF_6 (0.11 g, 0.67 mmol, 10 equiv) was added. The mixture was vigorously stirred at RT for 13 h and two phases were separated. The aqueous phase was extracted with CH_2Cl_2 (3x). The combined organic phases were dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue **S9-PF$_6$** was directly used for the crystal growth (*vide infra*).

3. X-ray structure of [2]rotaxane S9-PF$_6$



S9-PF$_6$

Single crystals of **S9-PF$_6$** were obtained by slow evaporation from a solution in EtOAc/MeOH/CH_2Cl_2 at 23 °C. A suitable crystal was selected and the sample was measured on a 'Bruker/Nonius Kappa Apex2' diffractometer. The crystal was kept at 100.0(2) K during data collection. Using Olex2[5], the structure was solved with the XT[6] structure solution program using Direct Methods and refined with the XL[7] refinement package using Least Squares minimisation.

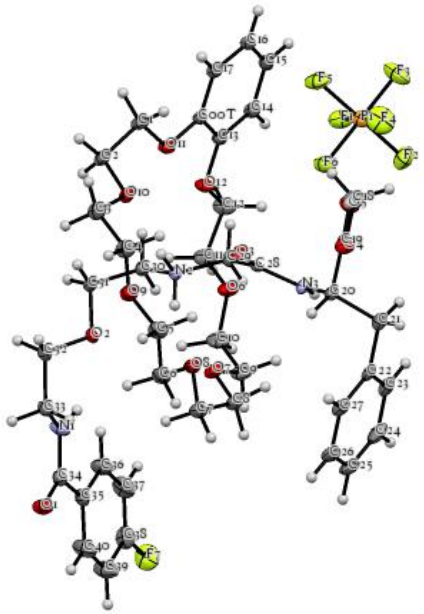


Figure S1. ORTEP diagram of **S9-PF_6**

Table S1 Crystal data and structure refinement for jb040915_1_1_0m.

Identification code	jb040915_1_1_0m
Empirical formula	$C_{41}H_{57}F_7N_3O_{12}P$
Formula weight	947.86
Temperature/K	100.0(2)
Crystal system	monoclinic
Space group	$P2_1$
a/Å	9.9986(15)
b/Å	18.897(3)
c/Å	12.0500(19)
α/°	90
β/°	91.502(6)
γ/°	90
Volume/$Å^3$	2276.0(6)
Z	2
$\rho_{calc}g/cm^3$	1.383
μ/mm^{-1}	0.152
F(000)	996.0
Crystal size/mm^3	0.26 × 0.16 × 0.08
Radiation	MoKα (λ = 0.71073)
2Θ range for data collection/°	5.228 to 55.088
Index ranges	-10 ≤ h ≤ 13, -24 ≤ k ≤ 23, -15 ≤ l ≤ 15
Reflections collected	24488
Independent reflections	10336 [R_{int} = 0.0319, R_{sigma} = 0.0500]
Data/restraints/parameters	10336/5/590
Goodness-of-fit on F^2	1.013
Final R indexes [I>=2σ (I)]	R_1 = 0.0366, wR_2 = 0.0686
Final R indexes [all data]	R_1 = 0.0486, wR_2 = 0.0732
Largest diff. peak/hole / e $Å^{-3}$	0.21/-0.29
Flack parameter	0.06(5)

Table S2 Bond Lengths for jb040915_1_1_0m.

Atom	Atom	Length/Å	Atom	Atom	Length/Å
F7	C38	1.362(3)	O6	C10	1.419(3)
O1	C34	1.232(3)	O6	C11	1.421(3)

O2	C31	1.420(3)	O7	C8	1.420(3)
O2	C32	1.427(3)	O7	C9	1.418(3)
O3	C28	1.221(3)	O8	C6	1.435(3)
O4	C18	1.447(3)	O8	C7	1.424(3)
O4	C19	1.338(3)	O9	C4	1.425(3)
O5	C19	1.201(3)	O9	C5	1.425(3)
N1	C33	1.450(4)	O10	C2	1.426(3)
N1	C34	1.341(3)	O10	C3	1.429(3)
N2	C29	1.485(3)	O11	C00T	1.375(3)
N2	C30	1.491(3)	O11	C1	1.428(3)
N3	C20	1.452(3)	O12	C12	1.433(3)
N3	C28	1.345(3)	O12	C13	1.368(3)
C19	C20	1.528(4)	C00T	C13	1.400(4)
C20	C21	1.523(4)	C00T	C17	1.383(4)
C21	C22	1.512(4)	C1	C2	1.499(4)
C22	C23	1.388(4)	C3	C4	1.501(4)
C22	C27	1.396(4)	C5	C6	1.489(4)
C23	C24	1.381(4)	C7	C8	1.489(4)
C24	C25	1.375(4)	C9	C10	1.494(4)
C25	C26	1.383(4)	C11	C12	1.489(4)
C26	C27	1.392(4)	C13	C14	1.383(3)
C28	C29	1.514(3)	C14	C15	1.392(4)
C30	C31	1.498(3)	C15	C16	1.374(4)
C32	C33	1.500(4)	C16	C17	1.395(4)
C34	C35	1.495(4)	P1	F1	1.6019(17)
C35	C36	1.387(4)	P1	F2	1.6068(19)
C35	C40	1.386(4)	P1	F3	1.5872(18)
C36	C37	1.382(4)	P1	F4	1.6012(19)
C37	C38	1.355(4)	P1	F5	1.5992(19)
C38	C39	1.371(4)	P1	F6	1.5995(17)
C39	C40	1.384(4)			

Table S3 Bond Angles for jb040915_1_1_0m.

Atom	Atom	Atom	Angle/°	Atom	Atom	Atom	Angle/°
C31	O2	C32	111.21(19)	C7	O8	C6	110.4(2)
C19	O4	C18	114.5(2)	C4	O9	C5	111.2(2)
C34	N1	C33	120.3(2)	C2	O10	C3	111.94(19)
C29	N2	C30	113.73(19)	C00T	O11	C1	115.6(2)
C28	N3	C20	118.2(2)	C13	O12	C12	116.0(2)
O4	C19	C20	110.4(2)	O11	C00T	C13	116.4(2)
O5	C19	O4	124.1(2)	O11	C00T	C17	124.0(2)
O5	C19	C20	125.6(2)	C17	C00T	C13	119.6(2)
N3	C20	C19	109.6(2)	O11	C1	C2	110.1(2)
N3	C20	C21	110.0(2)	O10	C2	C1	110.6(2)
C21	C20	C19	111.36(19)	O10	C3	C4	108.7(2)
C22	C21	C20	112.0(2)	O9	C4	C3	108.4(2)
C23	C22	C21	121.0(2)	O9	C5	C6	109.4(2)
C23	C22	C27	118.2(3)	O8	C6	C5	110.3(2)
C27	C22	C21	120.8(2)	O8	C7	C8	110.4(2)
C24	C23	C22	121.4(3)	O7	C8	C7	110.4(2)
C25	C24	C23	120.2(3)	O7	C9	C10	110.3(2)
C24	C25	C26	119.5(3)	O6	C10	C9	109.7(2)
C25	C26	C27	120.5(3)	O6	C11	C12	109.4(2)
C26	C27	C22	120.3(3)	O12	C12	C11	108.6(2)
O3	C28	N3	122.8(2)	O12	C13	C00T	116.1(2)

O3	C28	C29	121.3(2)	O12	C13	C14	124.3(2)
N3	C28	C29	115.9(2)	C14	C13	C00T	119.6(2)
N2	C29	C28	107.92(19)	C13	C14	C15	120.4(3)
N2	C30	C31	109.7(2)	C16	C15	C14	120.0(3)
O2	C31	C30	107.7(2)	C15	C16	C17	120.1(3)
O2	C32	C33	108.1(2)	C00T	C17	C16	120.3(3)
N1	C33	C32	111.2(2)	F1	P1	F2	89.53(10)
O1	C34	N1	121.2(3)	F3	P1	F1	90.04(10)
O1	C34	C35	120.5(2)	F3	P1	F2	90.43(11)
N1	C34	C35	118.2(2)	F3	P1	F4	90.89(11)
C36	C35	C34	123.4(3)	F3	P1	F5	90.05(10)
C40	C35	C34	118.0(2)	F3	P1	F6	179.62(12)
C40	C35	C36	118.6(3)	F4	P1	F1	178.86(12)
C37	C36	C35	120.5(3)	F4	P1	F2	89.80(10)
C38	C37	C36	118.9(3)	F5	P1	F1	90.13(10)
F7	C38	C39	118.5(3)	F5	P1	F2	179.41(11)
C37	C38	F7	118.5(3)	F5	P1	F4	90.53(11)
C37	C38	C39	123.0(3)	F5	P1	F6	89.92(9)
C38	C39	C40	117.6(3)	F6	P1	F1	89.58(9)
C39	C40	C35	121.4(3)	F6	P1	F2	89.59(10)
C10	O6	C11	111.0(2)	F6	P1	F4	89.49(10)
C9	O7	C8	110.9(2)				

Table S4 Torsion Angles for jb040915_1_1_0m.

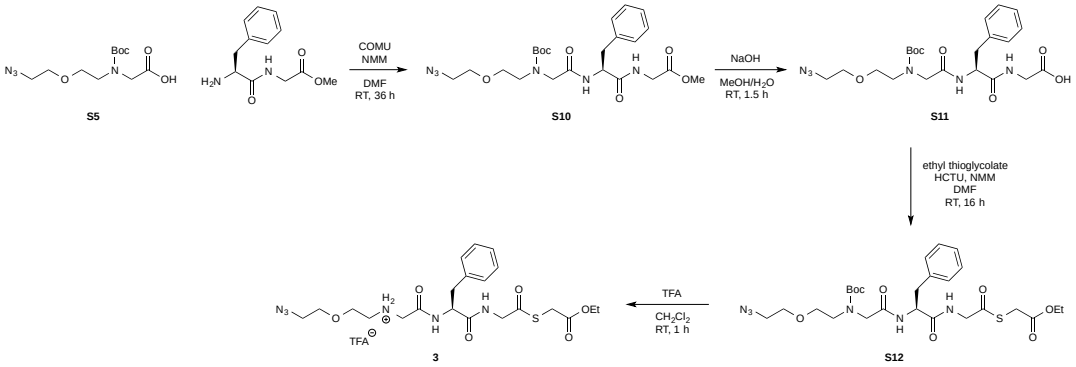
A	B	C	D	Angle/˚	A	B	C	D	Angle/˚
F7	C38	C39	C40	179.8(3)	C35	C36	C37	C38	0.2(5)
O1	C34	C35	C36	179.7(3)	C36	C35	C40	C39	0.4(4)
O1	C34	C35	C40	-2.0(4)	C36	C37	C38	F7	-180.0(3)
O2	C32	C33	N1	-53.3(3)	C36	C37	C38	C39	0.3(5)
O3	C28	C29	N2	-41.2(3)	C37	C38	C39	C40	-0.4(5)
O4	C19	C20	N3	147.1(2)	C38	C39	C40	C35	0.1(5)
O4	C19	C20	C21	-91.0(2)	C40	C35	C36	C37	-0.5(4)
O5	C19	C20	N3	-33.7(3)	O6	C11	C12	O12	-71.4(3)
O5	C19	C20	C21	88.2(3)	O7	C9	C10	O6	76.2(3)
N1	C34	C35	C36	-1.6(4)	O8	C7	C8	O7	-70.6(3)
N1	C34	C35	C40	176.7(2)	O9	C5	C6	O8	70.5(3)
N2	C30	C31	O2	-63.3(3)	O10	C3	C4	O9	-70.0(3)
N3	C20	C21	C22	-60.4(3)	O11	C00T	C13	O12	-1.9(3)
N3	C28	C29	N2	138.4(2)	O11	C00T	C13	C14	178.0(2)
C18	O4	C19	O5	1.7(4)	O11	C00T	C17	C16	-179.1(2)
C18	O4	C19	C20	-179.1(2)	O11	C1	C2	O10	71.9(3)
C19	C20	C21	C22	177.9(2)	O12	C13	C14	C15	-178.6(2)
C20	N3	C28	O3	-1.4(4)	C00T	O11	C1	C2	175.6(2)
C20	N3	C28	C29	179.0(2)	C00T	C13	C14	C15	1.5(4)
C20	C21	C22	C23	121.7(3)	C1	O11	C00T	C13	-168.9(2)
C20	C21	C22	C27	-57.9(3)	C1	O11	C00T	C17	11.3(3)
C21	C22	C23	C24	179.7(2)	C2	O10	C3	C4	-177.8(2)
C21	C22	C27	C26	179.3(2)	C3	O10	C2	C1	148.1(2)
C22	C23	C24	C25	1.1(4)	C4	O9	C5	C6	-178.9(2)
C23	C22	C27	C26	-0.3(4)	C5	O9	C4	C3	177.0(2)
C23	C24	C25	C26	-0.5(4)	C6	O8	C7	C8	-174.3(2)
C24	C25	C26	C27	-0.5(4)	C7	O8	C6	C5	175.7(2)
C25	C26	C27	C22	0.9(4)	C8	O7	C9	C10	178.8(2)
C27	C22	C23	C24	-0.7(4)	C9	O7	C8	C7	-166.0(2)
C28	N3	C20	C19	-62.5(3)	C10	O6	C11	C12	-168.4(2)

C28	N3	C20	C21	174.8(2)	C11	O6	C10	C9	-178.3(2)
C29	N2	C30	C31	178.6(2)	C12	O12	C13	C00T	179.2(2)
C30	N2	C29	C28	179.9(2)	C12	O12	C13	C14	-0.7(3)
C31	O2	C32	C33	166.2(2)	C13	O12	C12	C11	179.3(2)
C32	O2	C31	C30	173.7(2)	C13	C00T	C17	C16	1.2(4)
C33	N1	C34	O1	8.1(4)	C13	C14	C15	C16	0.3(4)
C33	N1	C34	C35	-170.6(2)	C14	C15	C16	C17	-1.4(4)
C34	N1	C33	C32	-176.1(2)	C15	C16	C17	C00T	0.7(4)
C34	C35	C36	C37	177.8(3)	C17	C00T	C13	O12	177.8(2)
C34	C35	C40	C39	-178.0(3)	C17	C00T	C13	C14	-2.3(4)

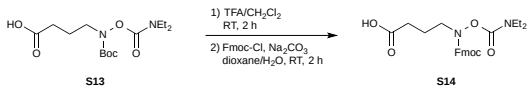
4. Synthesis of lasso peptide L1

4.1. Synthesis of [2]rotaxane 4

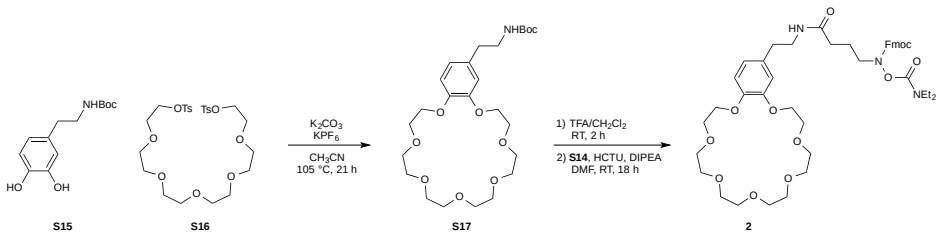
a)



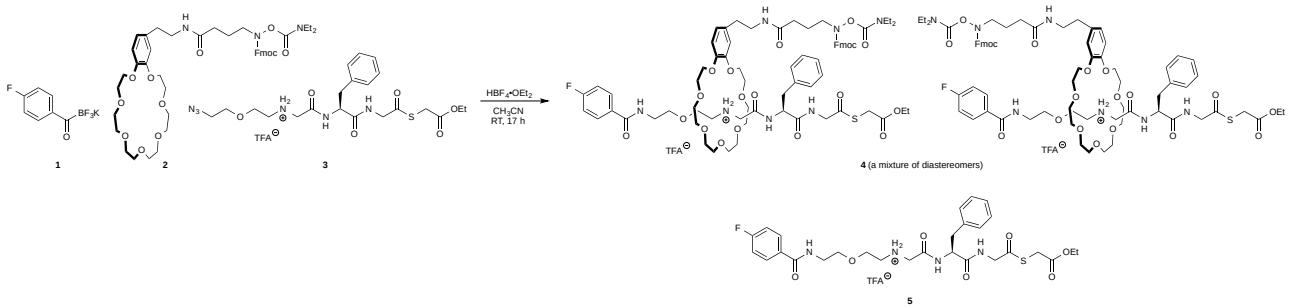
b)



c)

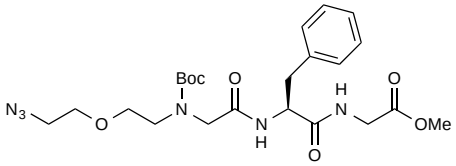


d)



Scheme S2. Synthesis of [2]rotaxane **4**

Azide-Phe-Gly methyl ester S10



S10

Azide-acid **S5** (1.5 g, 5.2 mmol, 1.0 equiv), COMU (2.2 g, 5.2 mmol, 1.0 equiv), and NMM (1.1 mL, 10 mmol, 2.0 equiv) were premixed in DMF (10 mL), and to this mixture was added a solution of H_2N-Phe-Gly-OMe[8] (1.2 g, 5.2 mmol, 1.0 equiv) in DMF (5.0 mL). The mixture was stirred at RT for 36 h and diluted with CH_2Cl_2 and H_2O. The aqueous phase was acidified with 1 M aq HCl to pH 3 and extracted with CH_2Cl_2 (3x). The combined organic phases were washed with brine, dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was purified by flash column chromatography (hexanes/EtOAc 1:2) to give **S10** (2.3 g, 87% yield) as a pale yellow amorphous.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.28-7.24 (m, 2H), 7.21-7.18 (m, 3H), 7.03-7.00 (m, 1H), 6.76-6.65 (m, 1H), 4.75 (m, 1H), 4.01-3.84 (m, 4H), 3.68-3.67 (m, 3H), 3.57-3.30 (m, 8H), 3.11-3.09 (m, 2H), 1.39 (s, 9H).

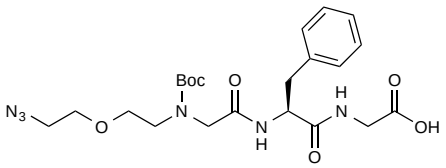
^{13}C NMR (100 MHz, $CDCl_3$) δ 171.13, 169.82, 169.75, 155.98, 155.13, 136.59, 129.26, 128.61, 126.92, 81.08, 69.47, 69.26, 54.03, 53.82, 52.88, 52.20, 50.59, 48.67, 48.21, 41.15, 38.04, 37.34, 28.22.

$[\alpha]_D^{28}$ = –22.2° (c 0.24, $CHCl_3$)

IR (thin film) 3296, 2977, 2932, 2109, 1754, 1697, 1655, 1547, 1248, 1207, 1175 cm^{-1}.

HRMS (ESI) calcd for $C_{23}H_{35}N_6O_7$ $[M+H]^+$: 507.2562, found: 507.2560.

Azide-Phe-Gly acid S11



S11

To a solution of **S10** (2.0 g, 3.9 mmol, 1.0 equiv) in MeOH (12 mL) was added 1 M aq NaOH (5.9 mL, 5.9 mmol, 1.5 equiv). The mixture was stirred at RT for 1.5 h and diluted with CH_2Cl_2 and H_2O. The aqueous phase was acidified with 1 M aq HCl to pH 2 and extracted with CH_2Cl_2 (3x). The combined organic phases were washed with brine, dried over Na_2SO_4, filtered and concentrated under reduced pressure to give **S11** (1.6 g, 83% yield) as a pale yellow solid.

^{1}H NMR (400 MHz, CD_3CN) δ 7.31-7.01 (m, 7H), 4.64-4.61 (m, 1H), 3.87 (d, *J* = 5.6 Hz, 2H), 3.79 (s, 2H), 3.60-3.51 (m, 4H), 3.36-3.28 (m, 4H), 3.23-3.13 (m, 1H), 2.95-2.89 (m, 1H), 1.42-1.33 (m, 9H).

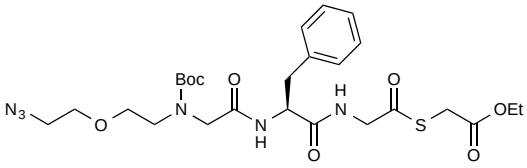
^{13}C NMR (100 MHz, CD_3CN) δ 172.51, 171.27, 170.83, 156.88, 156.16, 138.43, 130.28, 129.35, 127.60, 81.30, 80.88, 70.19, 69.73, 55.03, 52.57, 51.43, 49.03, 48.34, 41.69, 38.60, 38.06, 28.48.

$[\alpha]_D^{27}$ = –15.8° (c 0.23, $CHCl_3$)

IR (thin film) 3305, 2977, 2932, 2110, 1660, 1540, 1406, 1250, 1146 cm^{-1}.

HRMS (MALDI) calcd for $C_{22}H_{33}N_6O_7$ $[M+H]^+$: 493.2405, found: 493.2405.

Azide-Phe-Gly thioester S12



S12

Azide-acid **S11** (1.6 g, 3.2 mmol, 1.0 equiv), HCTU (1.3 g, 3.2 mmol, 1.0 equiv), and NMM (0.70 mL, 6.4 mmol, 2.0 equiv) were premixed in DMF (5.0 mL), and to this mixture was added ethyl thioglycolate (0.42 mL, 3.8 mmol, 1.2 equiv). The mixture was stirred at RT for 16 h and diluted with CH_2Cl_2 and H_2O. The aqueous phase was extracted with CH_2Cl_2 (3x). The combined organic phases were washed with H_2O/brine (1:1, 3x), dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was purified by flash column chromatography (hexanes/EtOAc 1:2) to give **S12** (0.84 g, 44% yield) as a pale yellow amorphous.

^{1}H NMR (400 MHz, CD_3CN) δ 7.54-7.20 (m, 6H), 6.99 (br s, 1H), 4.70-4.66 (m, 1H), 4.13 (q, *J* = 7.1 Hz, 2H), 4.07 (d, *J* = 6.2 Hz, 2H), 3.79 (s, 2H), 3.65 (s, 2H), 3.59-3.51 (m, 4H), 3.36-3.31 (m, 4H), 3.27-3.17 (m, 1H), 2.94 (dd, *J* = 14.2, 9.1 Hz, 1H), 1.42-1.32 (m, 9H), 1.22 (t, *J* = 7.2 Hz, 3H).

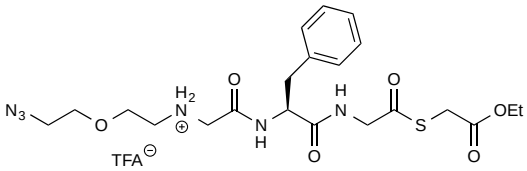
^{13}C NMR (100 MHz, CD_3CN) δ 197.78, 172.75, 170.61, 169.39, 156.89, 156.09, 138.49, 138.24, 130.21, 129.35, 127.57, 81.25, 80.71, 70.14, 69.69, 62.45, 55.10, 52.57, 51.40, 49.59, 49.02, 48.29, 38.33, 37.71, 31.63, 28.48, 14.41.

$[\alpha]_D^{28}$ = –23.7° (c 0.31, $CHCl_3$)

IR (thin film) 3289, 2977, 2932, 2108, 1697, 1657, 1542, 1298, 1249, 1170, 1146 cm^{-1}.

HRMS (ESI) calcd for $C_{26}H_{42}N_7O_8S$ $[M+NH_4]^+$: 612.2810, found: 612.2812.

Azide-Phe-Gly thioester TFA salt 3



3

To a solution of **S12** (0.34 g, 0.57 mmol) in CH_2Cl_2 (2.0 mL) was added TFA (2.0 mL). The mixture was stirred at RT for 1 h and diluted with CH_2Cl_2 and H_2O. The aqueous phase was extracted with CH_2Cl_2 (3x). The combined organic phases were dried over Na_2SO_4, filtered and concentrated

under reduced pressure to give **3** (0.31 g, 89% yield) as a pale yellow amorphous.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.08 (d, *J* = 7.9 Hz, 1H), 7.98 (t, *J* = 5.9 Hz, 1H), 7.73 (br s, 2H), 7.28-7.15 (m, 5H), 4.78 (m, 1H), 4.18-4.10 (m, 4H), 4.02 (d, *J* = 16.2 Hz, 1H), 3.83 (d, *J* = 16.2 Hz, 1H), 3.75-3.73 (m, 2H), 3.65 (d, *J* = 0.78 Hz, 2H), 3.62-3.60 (m, 2H), 3.37 (m, 2H), 3.22-3.13 (m, 3H), 2.95 (dd, *J* = 13.9, 8.6 Hz, 1H), 1.26 (t, *J* = 7.1 Hz, 3H).

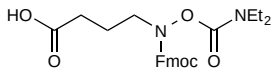
^{13}C NMR (100 MHz, $CDCl_3$) δ 195.49, 172.45, 169.29, 165.54, 160.84 (q, *J* = 39 Hz), 135.74, 129.18, 128.66, 127.22, 115.42 (q, *J* = 287 Hz), 69.84, 65.66, 62.40, 55.32, 50.46, 48.85, 48.54, 47.77, 37.82, 30.95, 13.89.

$[\alpha]_D^{26}$ = –12.3° (c 0.47, $CHCl_3$)

IR (thin film) 3287, 3067, 3033, 2987, 2932, 2109, 1667, 1556, 1304, 1200, 1179, 1139 cm^{-1}.

HRMS (ESI) calcd for $C_{21}H_{31}N_6O_6S$ $[M+H–TFA]^+$: 495.2020, found: 495.2015.

N-Fmoc hydroxylamine S14



S14

To a solution of *N*-Boc hydroxylamine **S13**[9] (1.2 g, 3.8 mmol, 1.0 equiv) in CH_2Cl_2 (3.0 mL) was added TFA (3.0 mL). The mixture was stirred at RT for 2 h and the volatiles were evaporated under reduced pressure. The residue was dissolved in dioxane/H_2O (10 mL, 1:1), and Na_2CO_3 (2.0 g, 19 mmol, 5.0 equiv) followed by Fmoc chloride (1.2 g, 4.6 mmol, 1.2 equiv) were added to this solution. The mixture was stirred at RT for 2 h and diluted with CH_2Cl_2. The aqueous phase was acidified with 1 M aq HCl to pH 2 and extracted with CH_2Cl_2 (3x). The combined organic phases were washed with brine, dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was purified by flash column chromatography (hexanes/EtOAc 1:2) to give **S14** (rotamers, 1.0 g, 60% yield over 2 steps) as a colorless oil that solidified in the freezer.

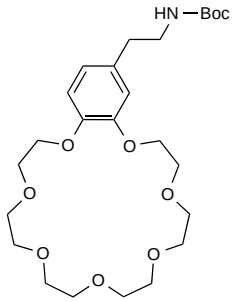
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.75 (m, 2H), 7.60-7.55 (m, 2H), 7.39 (m, 2H), 7.30 (m, 2H), 4.50 (d, *J* = 6.8 Hz, 2H), 4.24 (t, *J* = 6.8 Hz, 1H), 3.73-3.71 (m, 2H), 3.32-3.30 (m, 4H), 2.42 (t, *J* = 7.3 Hz, 2H), 1.90 (q, *J* = 6.9 Hz, 2H), 1.16 (t, *J* = 7.2 Hz, 6H).

^{13}C NMR (100 MHz, $CDCl_3$) δ 178.22, 155.80, 153.96, 143.59, 141.28, 127.72, 127.08, 124.96, 119.92, 68.14, 49.70, 47.04, 43.08, 41.60, 30.84, 22.24, 14.02, 13.26.

IR (thin film) 2978, 2933, 1741, 1714, 1450, 1422, 1270, 1145, 1109 cm^{-1}.

HRMS (ESI) calcd for $C_{24}H_{29}N_2O_6$ $[M+H]^+$: 441.2020, found: 441.2021.

B21C7-*N*-Boc amine S17



S17

To a solution of *N*-Boc dopamine **S15**[10] (1.6 g, 6.4 mmol, 1.0 equiv) in CH_3CN (50 mL) was added hexaethylene glycol ditosylate **S16**[4] (3.8 g, 6.4 mmol, 1.0 equiv), K_2CO_3 (2.7 g, 19 mmol, 3.0 equiv) followed by KPF_6 (1.8 g, 9.6 mmol, 1.5 equiv). The mixture was refluxed at 105 °C for 21 h and cooled to RT. The reaction mixture was diluted with H_2O and extracted with CH_2Cl_2 (3x). The combined organic phases were dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was purified by flash column chromatography (CH_2Cl_2/MeOH 20:1) to give **S17** (3.0 g, 94% yield) as a pale brown solid.

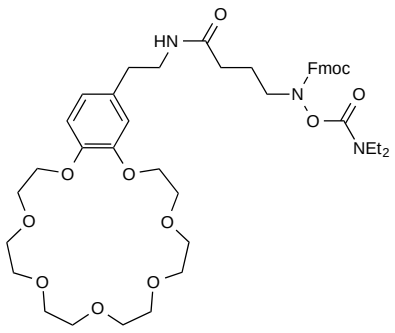
^{1}H NMR (400 MHz, $CDCl_3$) δ 6.84-6.82 (m, 1H), 6.74-6.72 (m, 2H), 4.58 (br s, 1H), 4.17-4.13 (m, 4H), 3.90-3.87 (m, 4H), 3.75-3.73 (m, 4H), 3.69-3.61 (m, 12H), 3.35-3.30 (m, 2H), 2.71 (t, *J* = 7.2 Hz, 2H), 1.42 (s, 9H).

^{13}C NMR (100 MHz, $CDCl_3$) δ 155.87, 148.47, 147.00, 132.73, 121.78, 114.83, 114.50, 79.17, 70.14, 70.03, 69.80, 69.34, 69.31, 68.81, 68.56, 41.81, 35.67, 28.38.

IR (thin film) 2927, 2875, 1702, 1514, 1260, 1169, 1121 cm^{-1}.

HRMS (ESI) calcd for $C_{25}H_{45}N_2O_9$ $[M+NH_4]^+$: 517.3120, found: 517.3123.

B21C7-*N*-Fmoc hydroxylamine 2



2

B21C7-*N*-Boc amine **S17** (0.49 g, 0.98 mmol) in CH_2Cl_2 (2.0 mL) was added TFA (2.0 mL). The mixture was stirred at RT for 2 h and basified with sat aq Na_2CO_3. The aqueous phase was extracted with CH_2Cl_2 (3x). The combined organic phases were dried over Na_2SO_4, filtered and concentrated under reduced pressure to give a crude material of the unprotected amine (0.34 g, 87% yield). The unprotected amine (0.34 g, 0.85 mmol, 1.0 equiv) was dissolved in DMF (2.0 mL),

and this solution was added to a solution of *N*-Fmoc hydroxylamine **S14** (0.45 g, 1.0 mmol, 1.2 equiv), HCTU (0.42 g, 1.0 mmol, 1.2 equiv), and DIPEA (0.44 mL, 2.6 mmol, 3.0 equiv) in DMF (3.0 mL). The mixture was stirred at RT for 18 h and diluted with CH_2Cl_2. The organic phase was washed with 1 M aq HCl (1x), then H_2O (2x), then dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was purified by preparative RP-HPLC using a YMC C18 column with a gradient of 40 to 95% CH_3CN in 28 min. The pure product fractions were pooled and lyophilized to obtain **2** (rotamers, 0.41 g, 59% yield) as a colorless amorphous.

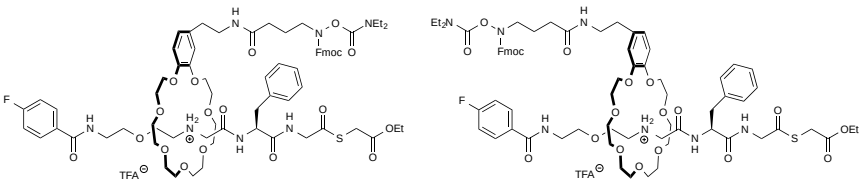
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.74 (d, *J* = 7.6 Hz, 2H), 7.56 (dd, *J* = 7.5, 1.0 Hz, 2H), 7.38 (t, *J* = 7.4 Hz, 2H), 7.29 (m, 2H), 6.80-6.78 (m, 1H), 6.73-6.69 (m, 2H), 6.50 (br s, 1H), 4.51 (d, *J* = 6.6 Hz, 2H), 4.22 (t, *J* = 6.6 Hz, 1H), 4.15-4.10 (m, 4H), 3.90-3.87 (m, 4H), 3.79-3.76 (m, 4H), 3.73-3.70 (m, 4H), 3.68-3.64 (m, 8H), 3.61 (t, *J* = 6.2 Hz, 2H), 3.47 (q, *J* = 6.8 Hz, 2H), 3.32-3.23 (m, 4H), 2.73 (t, *J* = 7.3 Hz, 2H), 2.24 (t, *J* = 7.3 Hz, 2H), 1.85 (p, *J* = 6.7 Hz, 2H), 1.15 (t, *J* = 7.1 Hz, 6H).

^{13}C NMR (100 MHz, $CDCl_3$) δ 174.07, 156.17, 154.14, 148.99, 147.55, 143.51, 141.32, 131.82, 127.81, 127.10, 124.93, 121.39, 120.00, 114.72, 114.51, 71.04, 71.03, 71.00, 70.98, 70.95, 70.51, 69.81, 69.79, 69.33, 69.20, 68.14, 49.65, 47.05, 43.15, 41.67, 41.06, 34.92, 33.01, 23.64, 14.06, 13.29.

IR (thin film) 2934, 2875, 1744, 1514, 1451, 1424, 1266, 1143, 1106 cm^{-1}.

HRMS (ESI) calcd for $C_{44}H_{63}N_4O_{12}$ $[M+NH_4]^+$: 839.4437, found: 839.4433.

[2]Rotaxane 4



4

4-Fluorophenyl KAT **1** (81 mg, 0.35 mmol, 1.0 equiv), crown ether **2** (0.29 g, 0.35 mmol, 1.0 equiv), and **3** (0.21 g, 0.35 mmol, 1.0 equiv) were dissolved in CH_3CN (3.2 mL), and HBF_4•OEt_2 (96 μL, 0.70 mmol, 2.0 equiv) was added. The mixture was stirred at RT for 17 h. The reaction was quenched with H_2O. The crude material was purified by preparative HPLC using a YMC C18 column with a gradient of 60 to 80% CH_3CN with 0.1% TFA in 28 min. The pure product fractions were pooled and lyophilized to obtain 116 mg of **4** (21% yield) as a mixture of diastereomers.

In the same purification, the peaks containing axle **5** were also collected, and these fractions were pooled and lyophilized. The lyophilizates were repurified by preparative HPLC using a YMC C18 column with a gradient of 20 to 95% CH_3CN with 0.1% TFA in 28 min. The pure product fractions were pooled and lyophilized to obtain 63 mg of **5** (31% yield).

^{1}H NMR (400 MHz, $CDCl_3$) δ 9.54 (dd, *J* = 16.1, 8.5 Hz, 1H), 9.00-8.94 (m, 1H), 7.86-7.63 (m, 6H), 7.56 (d, *J* = 7.4 Hz, 2H), 7.38 (t, *J* = 7.5 Hz, 2H), 7.34-7.27 (m, 4H), 7.19 (q, *J* = 7.6 Hz, 2H), 7.14-

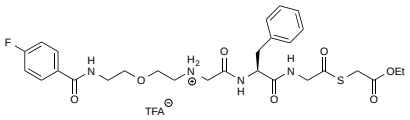
7.08 (m, 1H), 7.02-6.97 (m, 2H), 6.91 (q, *J* = 5.5 Hz, 1H), 6.68-6.53 (m, 4H), 4.80-4.64 (m, 1H), 4.45 (d, *J* = 6.9 Hz, 1H), 4.29-4.19 (m, 3H), 4.16-3.77 (m, 10H), 3.72-3.26 (m, 37H), 2.98-2.91 (m, 1H), 2.71 (t, *J* = 6.9 Hz, 2H), 2.22 (q, *J* = 6.9 Hz, 2H), 1.90-1.83 (m, 2H), 1.25-1.21 (m, 3H), 1.15 (t, *J* = 7.1 Hz, 6H).

^{13}C NMR (100 MHz, $CDCl_3$) δ 196.19, 196.14, 173.21, 173.15, 173.05, 168.62, 166.84, 166.09, 166.03, 164.59 (d, *J* = 252 Hz), 160.72 (q, *J* = 37.1 Hz), 156.10, 154.05, 146.83, 146.79, 145.47, 143.51, 141.24, 138.00, 132.20, 130.43 (d, *J* = 3.2 Hz), 129.53, 129.45, 129.21 (d, *J* = 9.0 Hz), 128.14, 127.79, 127.07, 126.18, 126.11, 124.96, 121.09, 119.96, 116.04 (q, *J* = 291 Hz),115.46 (d, *J* = 22.0 Hz), 112.17, 112.13, 111.38, 111.31, 71.15, 71.11, 71.09, 70.77, 70.67, 70.59, 70.54, 69.55, 69.46, 69.37, 69.35, 69.32, 68.19, 68.00, 67.91, 67.82, 66.33, 61.69, 56.09, 55.90, 49.76, 48.95, 48.44, 46.98, 46.35, 43.08, 41.61, 40.83, 40.76, 39.43, 38.00, 37.91, 34.93, 33.14, 30.81, 23.49, 14.06, 14.00, 13.27.

^{19}F NMR (376 MHz, $CDCl_3$) δ –107.82, –107.83.

IR (thin film) 3061, 2931, 1741, 1687, 1262, 1200, 1142, 1116 cm^{-1}.

HRMS (MALDI) calcd for $C_{72}H_{95}FN_7O_{19}S$ $[M+H–TFA]^+$: 1412.6382, found: 1412.6379.



5

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.29 (d, *J* = 8.2 Hz, 1H), 8.18 (t, *J* = 5.5 Hz, 1H), 7.85-7.81 (m, 2H), 7.58 (br s, 1H), 7.22-7.11 (m, 5H), 7.03 (t, *J* = 8.5 Hz, 2H), 4.79-4.73 (m, 1H), 4.14-4.05 (m, 4H), 3.95 (d, *J* = 16.1 Hz, 1H), 3.74 (d, *J* = 16.1 Hz, 1H), 3.65-3.57 (m, 8H), 3.16-3.06 (m, 3H), 2.88 (dd, *J* = 14.0, 8.9 Hz, 1H), 1.23 (t, *J* = 7.1 Hz, 3H).

^{13}C NMR (100 MHz, $CDCl_3$) δ 195.77, 171.96, 169.06, 167.48, 165.50, 164.77 (d, *J* = 252 Hz), 161.67 (q, *J* = 38.4 Hz), 136.21, 130.05 (d, *J* = 3.2 Hz), 129.70 (d, *J* = 8.9 Hz), 129.23, 128.52, 127.01, 116.14 (q, *J* = 291 Hz), 115.46 (d, *J* = 21.8 Hz), 70.08, 65.33, 62.22, 55.07, 48.84, 48.34, 47.42, 39.83, 37.95, 30.95, 13.95.

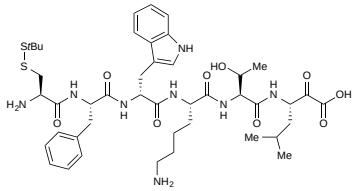
^{19}F NMR (376 MHz, $CDCl_3$) δ –107.85.

$[\alpha]_D^{28}$ = –10.3° (c 0.81, $CHCl_3$)

IR (thin film) 3293, 3066, 1669, 1555, 1504, 1296, 1200, 1133 cm^{-1}.

HRMS (ESI) calcd for $C_{28}H_{36}FN_4O_7S$ $[M+H–TFA]^+$: 591.2283, found: 591.2272.

4.2. Peptide α-ketoacid 7



7

Peptide α-ketoacid **7** was prepared using the protected leucine α-ketoacid resin **S1** on a 0.18 mmol scale (1.0 g) with a substitution capacity of 0.18 mmol/g. After the full assembly of amino acids, the resin was treated with (95:2.5:2.5) TFA:DODT:H_2O for 1 h and removed by filtration. The volatiles were evaporated from the filtrate under reduced pressure. The residue was triturated with Et_2O and centrifuged to obtain the crude **7**. Purification of crude **7** was performed by preparative HPLC using YMC C18 column (20 x 250 mm) with a gradient of 20 to 95% CH_3CN with 0.1% TFA in 28 min. The pure product fractions were pooled and lyophilized to obtain 44 mg of **7** (27% yield for peptide synthesis, resin cleavage and purification steps). Analytical HPLC and HRMS were used to confirm the purity and exact mass of the product.

HRMS (MALDI) calcd for $C_{44}H_{65}N_8O_9S_2$ $[M+H]^+$: 913.4310, found: 913.4319.

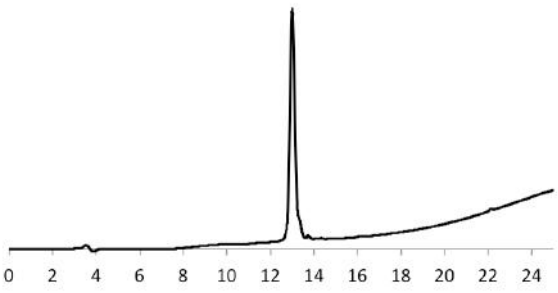


Fig. S2. Analytical HPLC of purified **7**

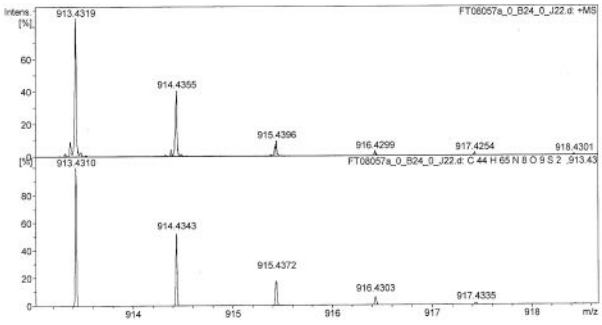
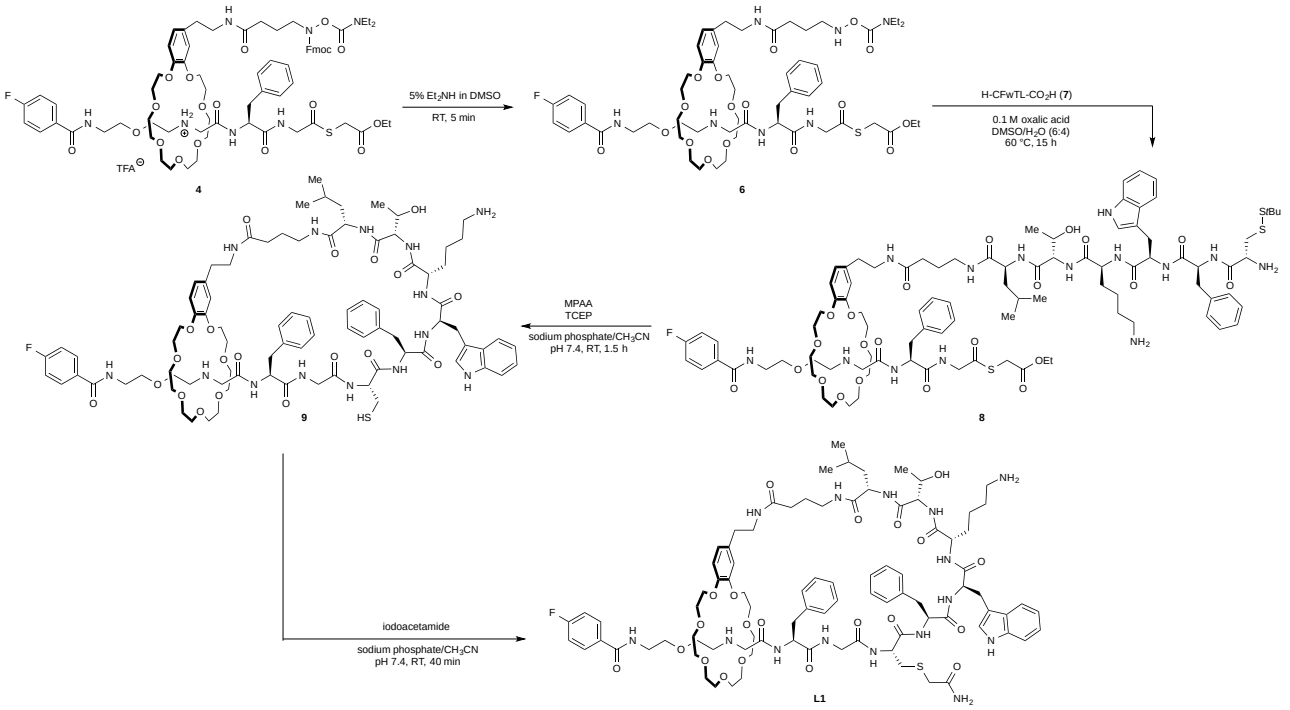


Fig. S3. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **7**

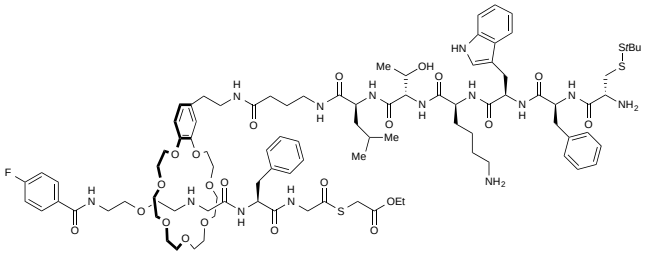
Note: Products derived from [2]rotaxane 4 were obtained as a mixture of diastereomers. For simplicity, strucutres of one of the diastereomers are shown in the following sections.

4.3. Synthesis of lasso peptide L1



Scheme S3. Synthesis of lasso peptide **L1**

Peptido[2]rotaxane 8



8

To a solution of rotaxane **4** (40 mg, 28 μmol) in DMSO (760 μL) was added Et_2NH (40 μL), and the mixture was incubated at RT for 5 min and purified by preparative HPLC using YMC C18 column (20 x 250 mm) with a gradient of 20 to 95% CH_3CN with 0.1% TFA in 28 min. The pure product fraction was lyophilized to give the unprotected hydroxylamine **6** (31 mg, 78% yield). The isolated unprotected hydroxylamine **6** was somewhat unstable and immediately used for the next ligation step. Characterization was therefore conducted only by HRMS (MALDI).

HRMS (MALDI) calcd for $C_{57}H_{85}FN_7O_{17}S$ $[M+H]^+$: 1190.5701, found: 1190.5701.

The lyophilizate **6** (21 mg, 22 μmol, 1.2 equiv) and peptide α-ketoacid **7** (17 mg, 18 μmol, 1.0 equiv) were dissolved in DMSO/H_2O (6:4, 360 μL, 0.1 M oxalic acid). The resulting mixture was incubated at 60 °C for 15 h and cooled to RT. Purification was performed by preparative HPLC

using YMC C18 column (20 x 250 mm) with a gradient of 20 to 95% CH_3CN with 0.1% TFA in 28 min. The pure product fractions were pooled and lyophilized to obtain 8.2 mg of **8** (23% yield). Analytical HPLC and HRMS were used to confirm the purity and exact mass of the product.

HRMS (MALDI) calcd for $C_{95}H_{138}FN_{14}O_{22}S_3$ $[M+H]^+$: 1941.9251, found: 1941.9277.

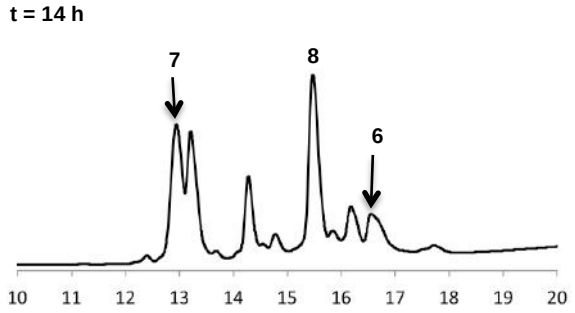


Fig. S4. HPLC monitoring of the KAHA ligation to form **8**

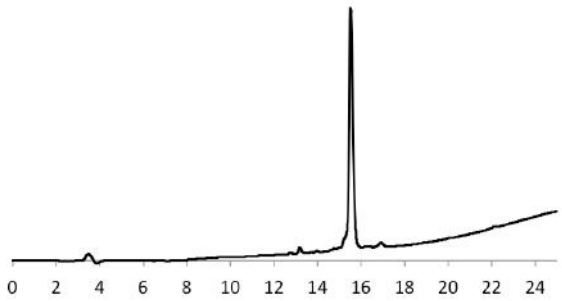


Fig. S5. Analytical HPLC of purified **8**

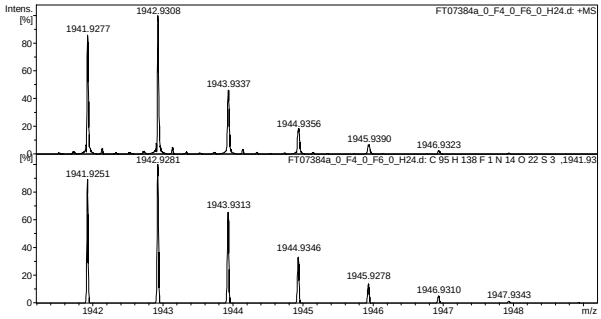
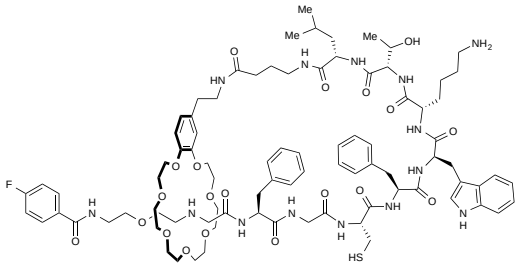


Fig. S6. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **8**

Lasso peptide 9



9

4-Mercaptophenylacetic acid (8.2 mg, 49 μmol, 10 equiv) and TCEP-HCl (28 mg, 98 μmol, 20 equiv) were dissolved in 0.25 M sodium phosphate buffer (pH 8.0)/CH_3CN (1:1, 1.2 mL), and pH of this solution was adjusted to 7.5 by adding 1 M aq NaOH. A solution of **8** (9.5 mg, 4.9 μmol, 1.0 equiv) in 0.1 M sodium phosphate buffer (pH 7.4)/CH_3CN (1:1, 0.20 mL) was prepared, and a portion (50 μL) of this solution was added to the ligation buffer. The mixture was incubated at RT for 10 min, and the next portion (50 μL) of the solution of **8** was added to the mixture. This addition-incubation process was repeated every 10 minutes. After complete addition, the mixture was further incubated at RT for 1 h and purified by preparative HPLC using YMC C18 column (20 x 250

mm) with a gradient of 20 to 95% CH_3CN with 0.1% TFA in 28 min. The pure product fractions were pooled and lyophilized to obtain 6.0 mg of **9** (71% yield). Analytical HPLC and HRMS were used to confirm the purity and exact mass of the product.

HRMS (MALDI) calcd for $C_{87}H_{122}FN_{14}O_{20}S$ $[M+H]^+$: 1733.8659, found: 1733.8661.

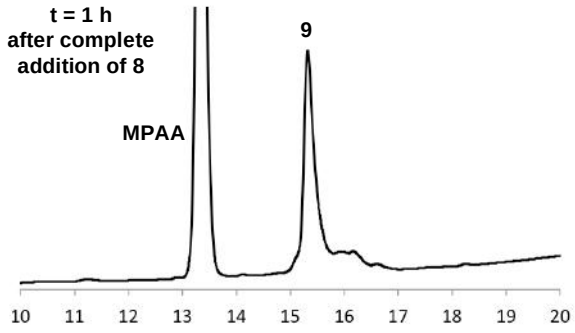


Fig. S7. HPLC of monitoring of the NCL to form **9**

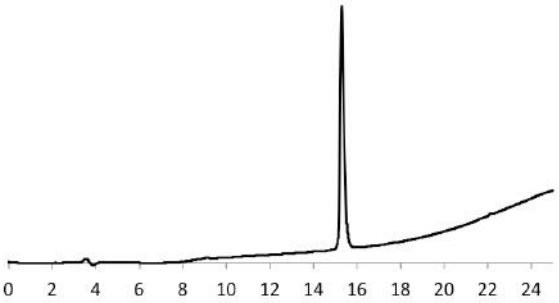


Fig. S8. Analytical HPLC of purified **9**

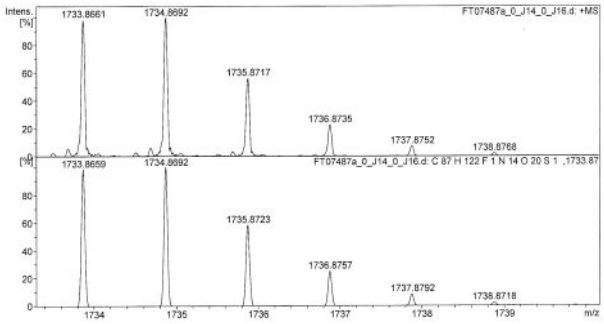
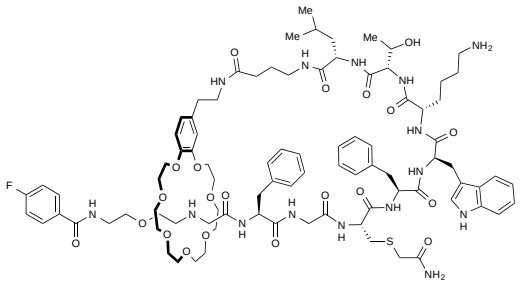


Fig. S9. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **9**

Cys-alkylated lasso peptide L1



L1

To a solution of **9** (8.9 mg, 5.1 μmol, 1.0 equiv) in 0.1 M sodium phosphate buffer (pH 7.4)/CH_3CN (1:1, 410 μL) was added a solution of iodoacetamide (1.0 mg, 5.6 μmol, 1.1 equiv) in 0.1 M sodium phosphate buffer (pH 7.4)/CH_3CN (1:1, 100 μL). The mixture was incubated at RT for 40 min and purified by preparative HPLC using YMC C18 column (20 x 250 mm) with a gradient of 20 to 95% CH_3CN with 0.1% TFA in 28 min. The pure product fractions were pooled and lyophilized to obtain 6.0 mg of **L1** (66% yield). Analytical HPLC and HRMS were used to confirm the purity and exact mass of the product.

HRMS (MALDI) calcd for $C_{89}H_{125}FN_{15}O_{21}S$ $[M+H]^+$: 1790.8874, found: 1790.8859.

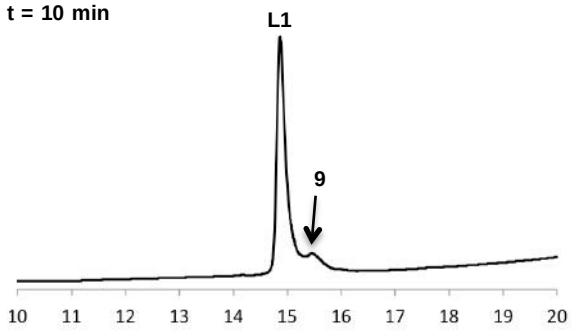


Fig. S10. HPLC monitoring of the cysteine alkylation to form **L1**

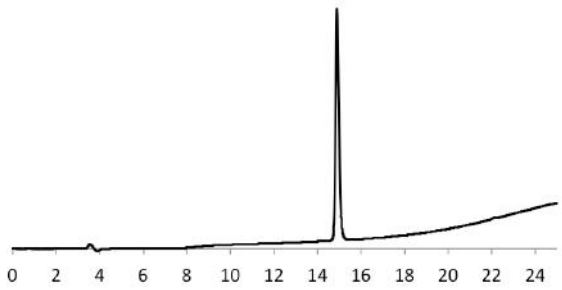


Fig. S11. Analytical HPLC of purified **L1**

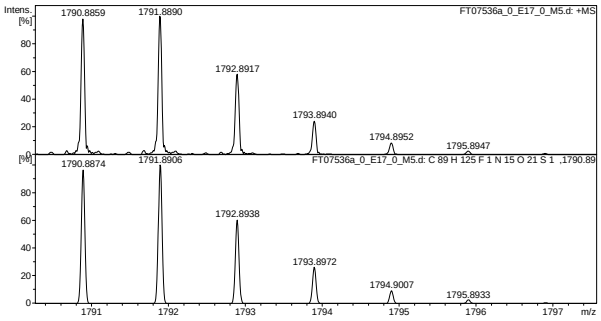
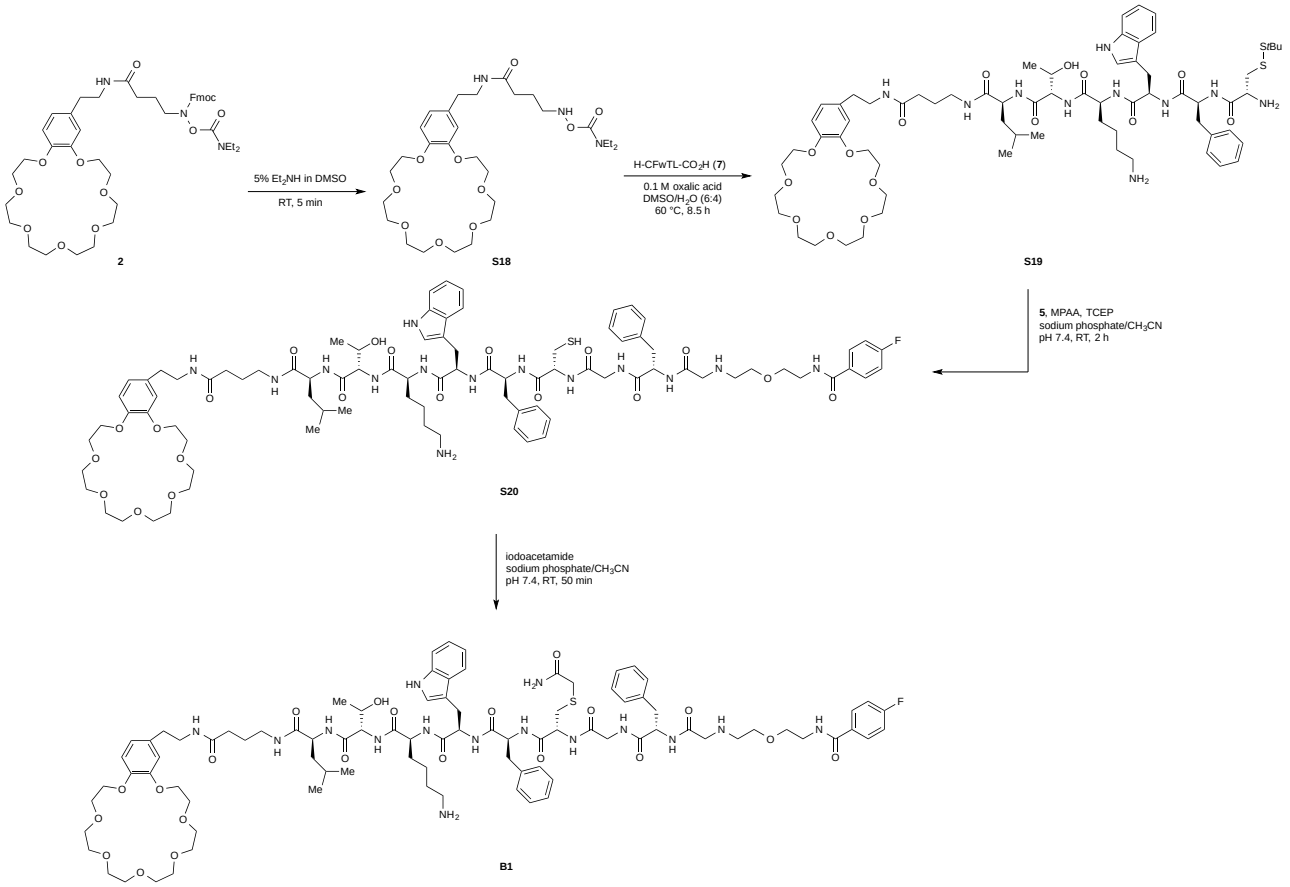


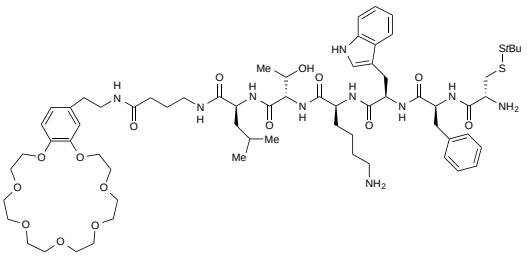
Fig. S12. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **L1**

5. Synthesis of branched-cyclic peptide B1



Scheme S4. Synthesis of branched-cyclic peptide **B1**

KAHA ligation product S19



S19

To a solution of crown ether **2** (91 mg, 0.11 mmol) in DMSO (570 μL) was added Et_2NH (30 μL), and the mixture was incubated at RT for 5 min and purified by preparative HPLC using YMC C18 column (20 x 250 mm) with a gradient of 20 to 95% CH_3CN with 0.1% TFA in 28 min. The pure product fraction was lyophilized to give the unprotected hydroxylamine **S18** (57 mg, 86% yield). The isolated unprotected hydroxylamine **S18** was somewhat unstable and immediately used for the next ligation step. Characterization was therefore conducted only by HRMS (ESI).

HRMS (ESI) calcd for $C_{29}H_{50}N_3O_{10}$ $[M+H]^+$: 600.3491, found: 600.3478.

The lyophilizate **S18** (14 mg, 23 μmol, 2.0 equiv) and peptide α-ketoacid **7** (11 mg, 12 μmol, 1.0 equiv) were dissolved in DMSO/H_2O (6:4, 234 μL, 0.1 M oxalic acid). The resulting mixture was incubated at 60 °C for 8.5 h and cooled to RT. Purification was performed by preparative HPLC

using YMC C18 column (20 x 250 mm) with a gradient of 20 to 95% CH_3CN with 0.1% TFA in 28 min. The pure product fractions were pooled and lyophilized to obtain 5.9 mg of **S19** (37% yield). Analytical HPLC and HRMS were used to confirm the purity and exact mass of the product.

HRMS (MALDI) calcd for $C_{67}H_{103}N_{10}O_{15}S_2$ $[M+H]^+$: 1351.7040, found: 1351.7039.

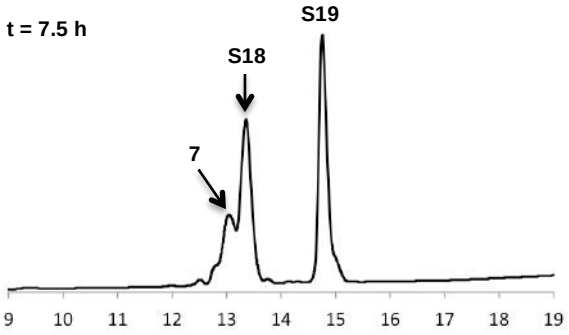


Fig. S13. HPLC monitoring of the KAHA ligation to form **S19**

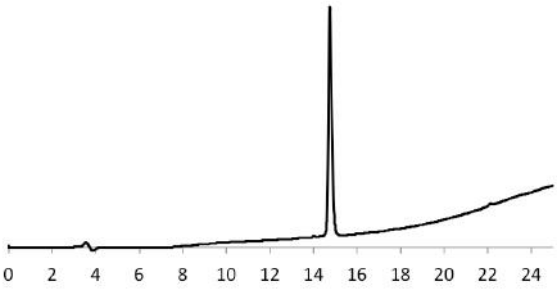


Fig. S14. Analytical HPLC of purified **S19**

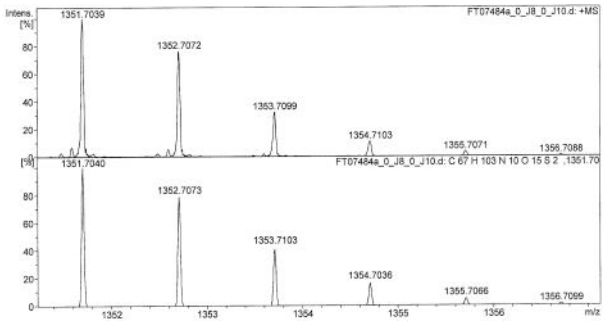
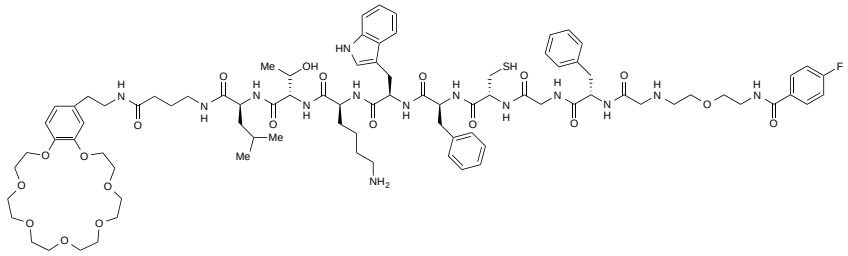


Fig. S15. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **S19**

NCL product S20



S20

4-Mercaptophenylacetic acid (12 mg, 73 μmol, 10 equiv) and TCEP-HCl (42 mg, 146 μmol, 20 equiv) were dissolved in 0.25 M sodium phosphate buffer (pH 8.0)/CH_3CN (1:1, 1.4 mL), and pH of this solution was adjusted to 7.9 by adding 1 M aq NaOH. To this ligation buffer were added a solution of axle-thioester **5** (5.2 mg, 8.8 μmol, 1.2 equiv) in CH_3CN (0.20 mL) followed by a solution of **S19** (9.9 mg, 7.3 μmol, 1.0 equiv) in 0.1 M sodium phosphate buffer (pH 7.4)/CH_3CN (1:1, 0.30 mL), and the mixture was incubated at RT for 2 h. Purification was performed by preparative HPLC using YMC C18 column (20 x 250 mm) with a gradient of 20 to 95% CH_3CN with 0.1% TFA in 28 min. The pure product fractions were pooled and lyophilized to obtain 9.2 mg of **S20** (73% yield).

Analytical HPLC and HRMS were used to confirm the purity and exact mass of the product.

HRMS (MALDI) calcd for $C_{87}H_{122}FN_{14}O_{20}S$ $[M+H]^+$: 1733.8659, found: 1733.8662.

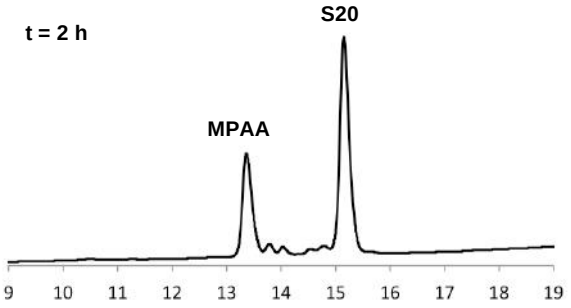


Fig. S16. HPLC monitoring of the NCL to form **S20**

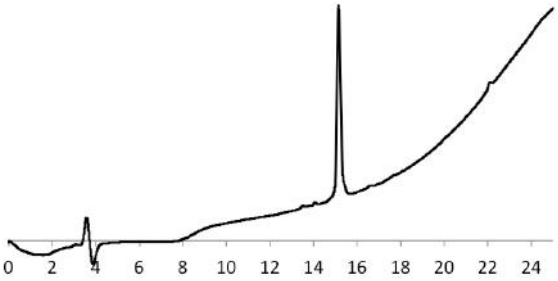


Fig. S17. Analytical HPLC of purified **S20**

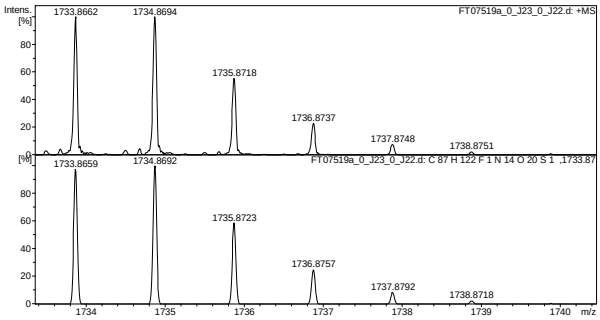
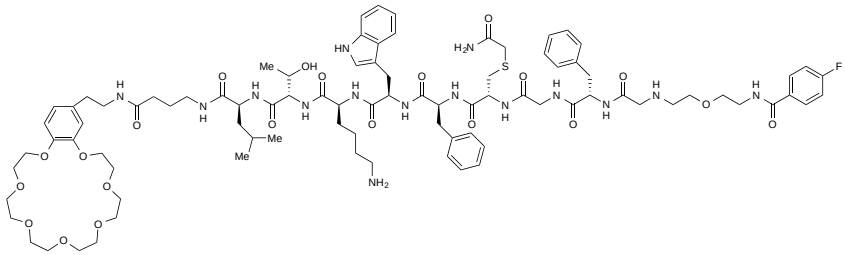


Fig. S18. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **S20**

Branched-cyclic peptide B1



B1

To a solution of **S20** (10 mg, 5.8 μmol, 1.0 equiv) in 0.1 M sodium phosphate buffer (pH 7.4)/CH_3CN (1:1, 0.46 mL) was added a solution of iodoacetamide (1.2 mg, 6.4 μmol, 1.1 equiv) in 0.1 M sodium phosphate buffer (pH 7.4)/CH_3CN (1:1, 0.12 mL). The mixture was incubated at RT for 50 min and purified by preparative HPLC using YMC C18 column (20 x 250 mm) with a gradient of 20 to 95% CH_3CN with 0.1% TFA in 28 min. The pure product fractions were pooled and lyophilized to obtain 8.1 mg of **B1** (78% yield). Analytical HPLC and HRMS were used to confirm the purity and exact mass of the product.

HRMS (MALDI) calcd for $C_{89}H_{125}FN_{15}O_{21}S$ $[M+H]^+$: 1790.8874, found: 1790.8871.

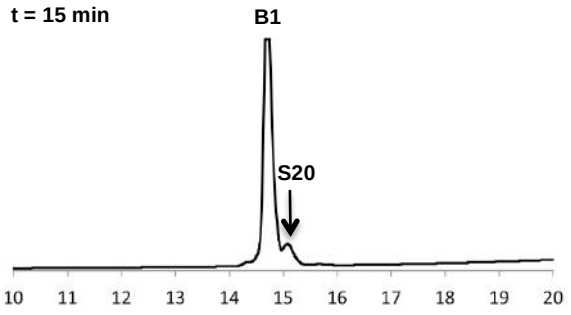


Fig. S19. HPLC monitoring of the cysteine alkylation to form **B1**

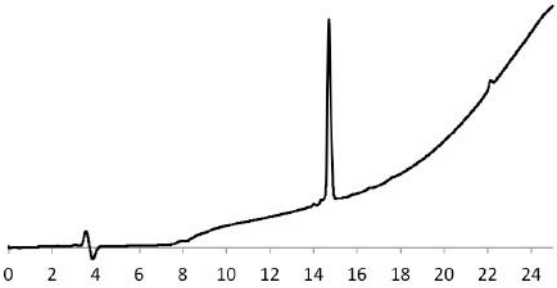


Fig. S20. Analytical HPLC of purified **B1**

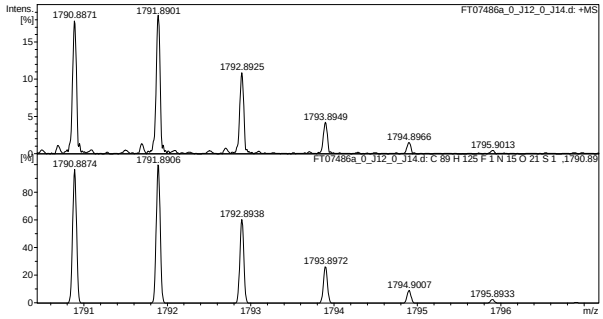


Fig. S21. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **B1**

6. Comparison of NMR spectra of L1 and B1

Significant peak overlaps around 3.2 ppm to 4.5 ppm in ^{1}H NMR made complete assignment impossible. However, ^{1}H NMR spectra of **L1** and **B1** were distinct, and a few characteristic peaks were found in the spectrum of **L1**.

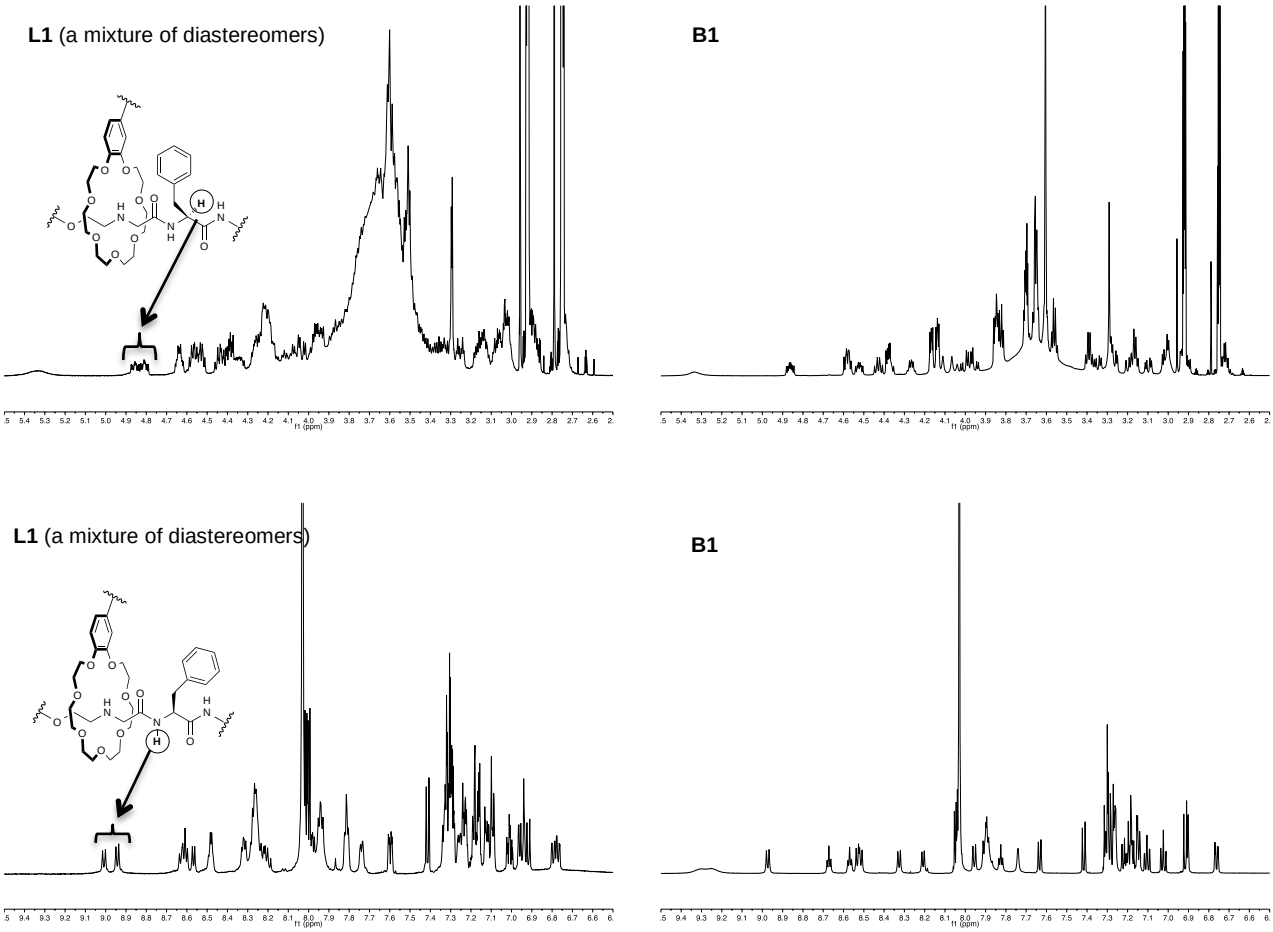
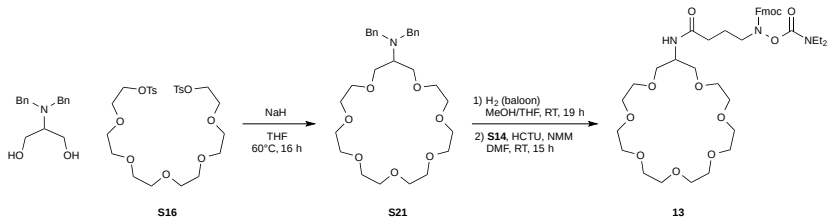


Figure S22. Selected regions of ^{1}H NMR spectra (600 MHz, d_7-DMF) of **L1** and **B1**

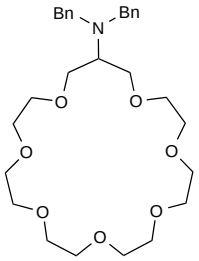
7. Synthesis of lasso peptide L2

7.1. Synthesis of crown ether-*N*-Fmoc hydroxylamine 13



Scheme S5. Synthesis of crown ether-*N*-Fmoc hydroxylamine **13**

Crown ether-diBn amine S21



S21

Note: this reaction was conducted under N_2 atmosphere. To a suspension of NaH (60% in mineral oil, 1.4 g, 36 mmol, 3.0 equiv) in dry THF (78 mL) was added a solution of 2-(*N*,*N*-dibenzylamino)-1,3-propanediol[11] (3.2 g, 12 mmol, 1.0 equiv) in dry THF (10 mL) at RT. The mixture was stirred at 60 °C for 10 min, and hexaethylene glycol ditosylate **S16** (7.0 g, 12 mmol, 1.0 equiv) was added. The reaction mixture was stirred at 60 °C for 16 h and cooled to RT. Sat aq NH_4Cl (10 mL) was added carefully, and the mixture was diluted with CH_2Cl_2 and H_2O. The two phases were separated and the aqueous phase was extracted with CH_2Cl_2 (3x). The combined organic phases were dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was purified by flash column chromatography (EtOAc to EtOAc/MeOH 50:1) to give **S21** (1.3 g, 21% yield) as a pale yellow oil.

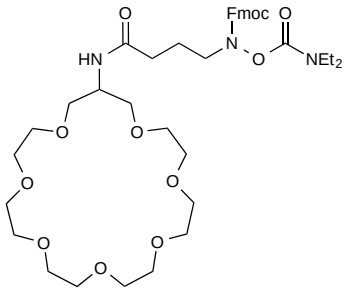
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.38-7.36 (m, 4H), 7.29-7.27 (m, 3H), 7.26-7.25 (m, 1H), 7.21-7.17 (m, 2H), 3.78 (s, 4H), 3.72-3.52 (m, 28H), 3.06 (p, *J* = 5.8 Hz, 1H).

^{13}C NMR (100 MHz, $CDCl_3$) δ 140.66, 128.50, 128.02, 126.58, 70.81, 70.75, 70.67, 70.62, 70.55, 69.71, 56.54, 55.22.

IR (thin film) 2866, 1494, 1453, 1351, 1297, 1250, 1115 cm^{-1}.

HRMS (ESI) calcd for $C_{29}H_{44}NO_7$ $[M+H]^+$: 518.3112, found: 518.3112.

Crown ether-*N*-Fmoc hydroxylamine 13



13

To a solution of **S21** (1.1 g, 2.1 mmol) in MeOH/THF (18 mL, 5:1) was added Pd/C (10%, 123 mg). The mixture was stirred at RT under 1 atm H_2 (baloon) for 19 h and filtered through Celite®. The filtrate was concentrated under reduced pressure.

The residue (0.76 g, 2.2 mmol, 1.0 equiv) was dissolved in DMF (2.0 mL) and added to a premixed solution of **S14** (1.1 g, 2.4 mmol, 1.1 equiv), HCTU (1.0 g, 2.4 mmol, 1.1 equiv) and NMM (0.48

mL, 4.4 mmol, 2.0 equiv) in DMF (8.0 mL). The mixture was stirred at RT for 15 h and diluted with CH_2Cl_2 and H_2O. The two phases were separated and the aqueous phase was extracted with CH_2Cl_2 (3x). The combined organic phases were washed with H_2O, dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was purified by flash column chromatography (EtOAc/MeOH 10:1) to give **13** (rotamers, 1.5 g, 88% yield) as a pale yellow oil.

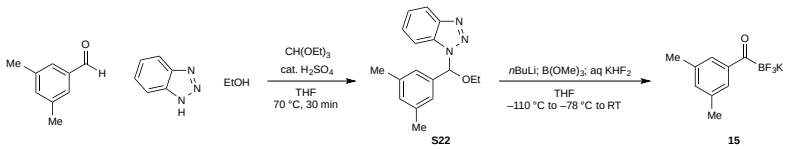
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.75 (d, *J* = 7.5 Hz, 2H), 7.58 (dd, *J* = 7.6, 1.1 Hz, 2H), 7.39 (td, *J* = 7.6, 1.0 Hz, 2H), 7.30 (td, *J* = 7.5, 1.2 Hz, 2H), 7.02-7.00 (m, 1H), 4.46 (d, *J* = 6.9 Hz, 2H), 4.25-4.18 (m, 2H), 3.71-3.60 (m, 28H), 3.53 (dd, *J* = 10.0, 5.2 Hz, 2H), 3.34-3.29 (m, 4H), 2.27 (t, *J* = 7.5 Hz, 2H), 1.96-1.89 (m, 2H), 1.16 (t, *J* = 7.1 Hz, 6H).

^{13}C NMR (100 MHz, $CDCl_3$) δ 172.87, 155.87, 153.92, 143.63, 141.24, 127.71, 127.06, 125.02, 119.90, 70.98, 70.63, 70.59, 70.56, 70.53, 70.45, 69.46, 68.08, 49.88, 49.19, 47.02, 42.99, 41.55, 33.10, 23.26, 14.06, 13.28.

IR (thin film) 2873, 1743, 1666, 1451, 1421, 1351, 1269, 1143, 1107 cm^{-1}.

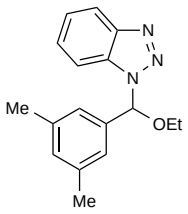
HRMS (ESI) calcd for $C_{39}H_{58}N_3O_{12}$ $[M+H]^+$: 760.4015, found: 760.4006.

7.2. Synthesis of 3,5-dimethylphenyl KAT 15



Scheme S6. Synthesis of 3,5-dimethylphenyl KAT **15**

Bt-ethoxy *N*,*O*-acetal S22



S22

3,5-Dimethylbenzaldehyde[12] (2.75 g, 20.5 mmol, 1.00 equiv), benzotriazole (3.05 g, 25.6 mmol, 1.25 equiv), EtOH (2.40 mL, 41.0 mmol, 2.00 equiv), and triethylorthoformate (10.2 mL, 61.5 mmol, 3.00 equiv) were dissolved in THF (40 mL) at RT. To this solution was added H_2SO_4 (10 drops), resulting in a white precipitate. The reaction was stirred at RT for 15 min, capped tightly with a plastic cap, and placed at 70 °C for 30 min. The reaction was cooled to RT, and 1.0 g solid $NaHCO_3$ was added and the heterogeneous solution was concentrated. The residue was purified by flash column chromatography (hexanes/EtOAc 10:1) to give **S22** (4.35 g, 75% yield) as a colorless oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.08-8.04 (m, 1H), 7.38-7.30 (m, 3H), 7.13 (s, 1H), 7.06-7.05 (m, 2H), 6.97-6.95 (m, 1H), 3.72 (dq, *J* = 9.5, 7.0 Hz, 1H), 3.45 (dq, *J* = 9.5, 7.0 Hz, 1H), 2.27 (s, 6H), 1.24

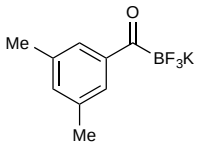
(t, *J* = 7.0 Hz, 3H).

^{13}C NMR (100 MHz, $CDCl_3$) δ 146.93, 138.17, 136.25, 131.11, 130.57, 127.31, 124.08, 123.57, 119.80, 111.77, 89.68, 64.94, 21.30, 14.67.

IR (thin film) 2977, 2918, 1609, 1449, 1331, 1274, 1163, 1104, 1085 cm^{-1}.

HRMS (ESI) calcd for $C_{17}H_{19}N_3NaO$ $[M+Na]^+$: 304.1420, found: 304.1423.

3,5-Dimethylphenyl KAT 15



15

Note: this reaction was conducted under N_2 atmosphere. In a 500 mL flask, **S22** (3.85 g, 13.7 mmol, 1.10 equiv) was dissolved in dry THF (88 mL) at RT. The flask was placed in a dry ice/acetone bath which was cooled with liq. N_2 until solidification of acetone (ca. –110 °C) and stirred for 15 min. *n*-BuLi (1.6 M in hexane, 8.1 mL, 12.5 mmol, 1.00 equiv) was added slowly down the side of the flask over 5 min. During this time, an intense green color developed. The solution was stirred for 2 min following the end of *n*-BuLi addition, and neat $B(OMe)_3$ (2.90 mL, 24.9 mmol, 2.00 equiv) was added slowly over 2 min directly into the solution. The deep green color gradually became brownish yellow after ca. 10 min of stirring. The flask was kept in the dry ice/acetone bath for 1 h, which slowly warmed to –78 °C, and the color of the reaction mixture became nearly colorless. The flask was removed from the bath, and with vigorous stirring, four portions of sat aq KHF_2 were added (4 x 10 mL, 40 mL total). As the reaction warmed, a biphasic mixture consisting of a milky white aqueous layer and a yellow organic layer formed. This was stirred overnight (16 h from the time of KHF_2 addition) over which time the organic layer stayed yellow. The two layers were separated and the yellow organic phase was evaporated until some water remained. To the residual yellow wet slurry were added Et_2O (30 mL) and hexanes (10 mL), and the mixture was stirred at RT for 1 h. The resulting precipitates were filtered, washed with Et_2O (4 x 10 mL) and dried to give **15** (1.19 g, 40% yield) as a white solid.

^{1}H NMR (600 MHz, d_6-acetone) δ 7.69-7.68 (m, 2H), 7.08-7.07 (m, 1H), 2.31-2.30 (m, 6H).

^{13}C NMR (150 MHz, d_6-acetone) δ 236.03, 137.67, 133.29, 127.13, 127.12, 21.36.

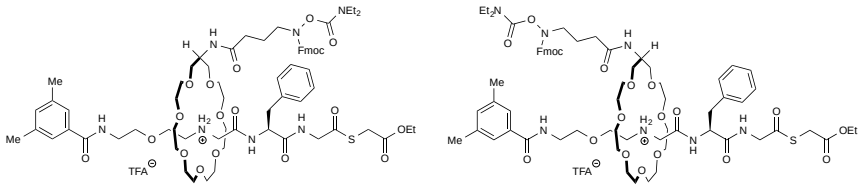
^{19}F NMR (470 MHz, d_6-acetone) δ –144.44.

^{11}B NMR (160 MHz, d_6-acetone) δ –0.82.

IR (thin film) 2957, 2901, 1637, 1594, 1299, 1200 cm^{-1}.

HRMS (ESI) calcd for $C_9H_9BF_3O$ $[M–K]^–$: 201.0706, found: 201.0707.

7.3. Synthesis of [2]Rotaxane S23



S23

3,5-Dimethylphenyl KAT **15** (0.13 g, 0.53 mmol, 1.1 equiv), crown ether **13** (0.37 g, 0.48 mmol, 1.0 equiv), and **3** (0.31 g, 0.48 mmol, 1.0 equiv) were dissolved in CH_3CN (2.4 mL), and HBF_4•OEt_2 (135 μL, 0.96 mmol, 2.0 equiv) was added. The mixture was stirred at RT for 16 h. The reaction was quenched with H_2O. The crude material was purified by preparative HPLC using a YMC C18 column with a gradient of 40 to 95% CH_3CN with 0.1% TFA in 38 min. The pure product fractions were pooled and lyophilized to obtain 91 mg of **S23** (14% yield) as a mixture of diastereomers.

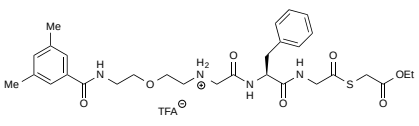
In the same purification, the peaks containing axle **S24** were also collected, and these fractions were pooled and lyophilized. The lyophilizates were repurified by preparative HPLC using a YMC C18 column with a gradient of 20 to 95% CH_3CN with 0.1% TFA in 28 min. The pure product fractions were pooled and lyophilized to obtain 114 mg of **S24** (34% yield).

^{1}H NMR (600 MHz, $CDCl_3$) δ 9.88 (d, *J* = 8.8 Hz, 1H x 0.55), 9.59 (d, *J* = 8.8 Hz, 1H x 0.45), 9.04 (t, *J* = 6.0 Hz, 1H x 0.45), 8.80 (t, *J* = 6.0 Hz, 1H x 0.45), 7.75-7.73 (m, 2H), 7.66-7.43 (m, 4H), 7.40-7.37 (m, 2H), 7.35-7.32 (m, 2H), 7.31-7.27 (m, 4H), 7.21-7.17 (m, 2H), 7.14-7.09 (m, 2H + 1H x 0.55), 6.95-6.93 (m, 1H), 6.59 (d, *J* = 8.5 Hz, 1H x 0.45), 4.90-4.83 (m, 1H), 4.43 (dd, *J* = 14.0, 7.0 Hz, 2H), 4.25-4.19 (m, 2H), 4.17-3.98 (m, 5H), 3.93-3.72 (m, 1H), 3.72-3.28 (m, 45H), 3.01-2.93 (m, 1H), 2.32-2.24 (m, 8H), 1.95-1.88 (m, 2H), 1.26-1.22 (m, 3H), 1.18-1.15 (m, 6H).

^{13}C NMR (150 MHz, $CDCl_3$) δ 196.10, 196.08, 173.13, 172.91, 172.72, 172.25, 168.60, 168.57, 168.55, 168.53, 165.78, 165.57, 161.20 (q, *J* = 35.7 Hz), 156.09, 155.87, 153.99, 153.97, 143.68, 143.53, 141.24, 141.23, 138.16, 138.13, 137.90, 134.74, 134.63, 133.10, 133.07, 129.40, 129.36, 128.19, 128.10, 127.79, 127.68, 127.07, 126.27, 126.06, 125.10, 124.97, 124.66, 119.97, 119.88, 116.40 (q, *J* = 292 Hz), 71.37, 71.32, 71.28, 71.15, 71.03, 71.01, 70.96, 70.90, 70.86, 70.84, 70.83, 70.80, 70.78, 70.75, 70.64, 70.60, 70.52, 70.51, 69.88, 69.76, 69.43, 69.39, 68.79, 68.73, 68.21, 68.19, 66.12, 66.03, 61.71, 61.68, 56.01, 55.84, 49.94, 49.69, 49.02, 49.00, 48.97, 48.27, 47.84, 47.13, 47.02, 46.96, 46.90, 43.06, 43.00, 41.59, 39.56, 39.41, 38.02, 37.95, 33.08, 33.00, 30.86, 23.38, 23.25, 21.22, 21.20, 14.04, 14.03, 13.29.

IR (thin film) 2908, 1741, 1688, 1539, 1452, 1422, 1200, 1092 cm^{-1}.

HRMS (MALDI) calcd for $C_{69}H_{98}N_7O_{19}S$ $[M+H–TFA]^+$: 1360.6633, found: 1360.6612.



S24

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.39 (d, *J* = 8.4 Hz, 1H), 8.26 (t, *J* = 6.5 Hz, 1H), 7.40 (s, 2H), 7.29 (br s, 1H), 7.21-7.11 (m, 5H), 7.08 (s, 1H), 4.77-4.71 (m, 1H), 4.11 (q, *J* = 7.2 Hz, 2H), 4.08-3.96 (m, 3H), 3.72-3.53 (m, 9H), 3.15 (dd, *J* = 14.0, 5.2 Hz, 1H), 3.08-3.00 (m, 2H), 2.86 (dd, *J* = 14.0, 9.3 Hz, 1H), 2.29 (s, 6H), 1.23 (t, *J* = 7.2 Hz, 3H).

^{13}C NMR (100 MHz, $CDCl_3$) δ 195.95, 171.89, 169.13, 168.85, 165.60, 162.02 (q, *J* = 34.3 Hz), 138.23, 136.45, 133.99, 133.27, 129.25, 128.46, 126.89, 124.92, 116.43 (q, *J* = 293 Hz), 70.22, 65.53, 62.15, 55.14, 48.80, 48.40, 47.29, 39.75, 37.82, 30.94, 21.10, 13.97.

$[\alpha]_D^{26}$ = –12.2° (c 0.32, $CHCl_3$)

IR (thin film) 3065, 2926, 1670, 1543, 1303, 1200, 1134 cm^{-1}.

HRMS (ESI) calcd for $C_{30}H_{41}N_4O_7S$ $[M+H–TFA]^+$: 601.2690, found: 601.2687.

7.4. Comparison of thermal stability of [2]rotaxanes

Initially, we have preprared [2]rotaxane **S25** using 4-fluorophenyl KAT **1** as a capping agent. Although **S25** was isolated and characterized by HRMS (MALDI), we found that **S25** was thermally unstable. **S25** gradually underwent deslippage under the KAHA ligation reaction condition (**60 °C**, DMSO/H_2O (6:4), 0.1 M oxalic acid). On the other hand, [2]rotaxane **S23** did not show deslippage under the same condition.

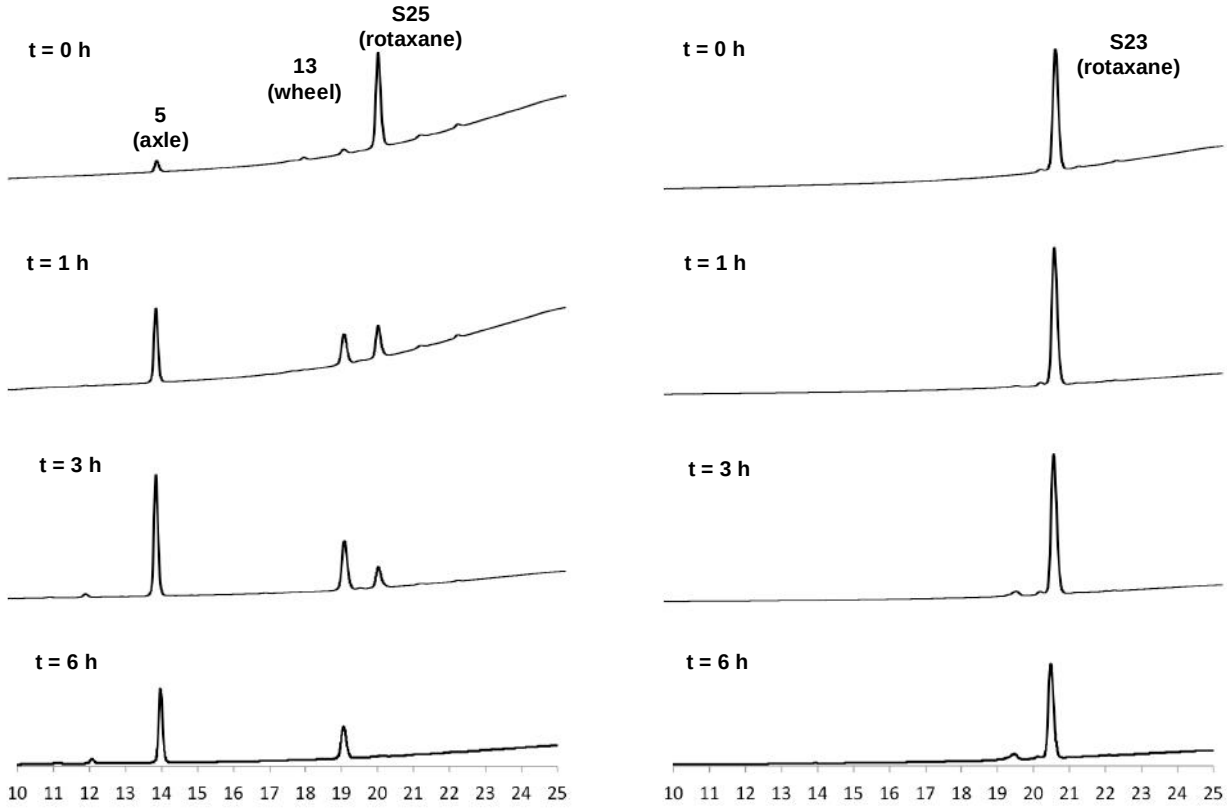
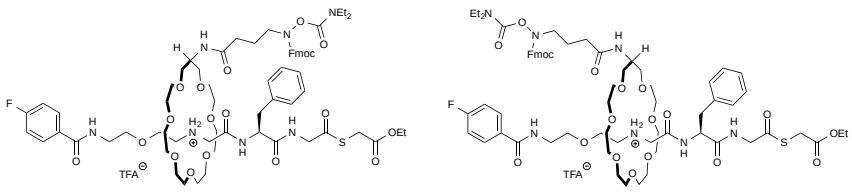


Figure S23. Comparison of thermal stability of [2]rotaxanes **S25** and **S23**

[2]Rotaxane S25



S25

4-Fluorophenyl KAT **1** (0.26 g, 1.1 mmol, 1.2 equiv), crown ether **13** (0.72 g, 0.95 mmol, 1.0 equiv), and **3** (0.47 g, 0.95 mmol, 1.0 equiv) were dissolved in CH_3CN (4.8 mL), and HBF_4•OEt_2 (264 μL, 1.9 mmol, 2.0 equiv) was added. The mixture was stirred at RT for 16 h. The reaction was quenched with H_2O. The crude material was purified by preparative HPLC using a YMC C18 column with a gradient of 40 to 95% CH_3CN with 0.1% TFA in 38 min. The pure product fractions were pooled and lyophilized to obtain 78 mg of **S25** (6% yield) as a mixture of diastereomers. Analytical HPLC and HRMS were used to confirm the purity and exact mass of the product.

HRMS (MALDI) calcd for $C_{67}H_{93}FN_7O_{19}S$ $[M+H–TFA]^+$: 1350.6225, found: 1350.6222.

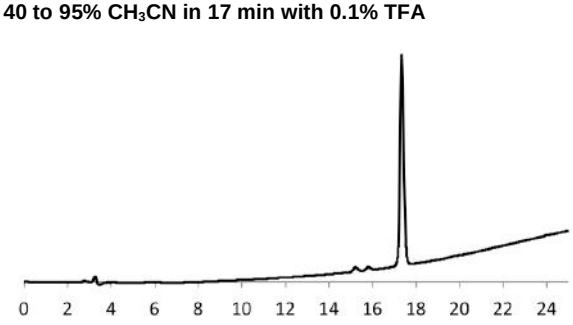


Fig. S24. Analytical HPLC of purified **S25**

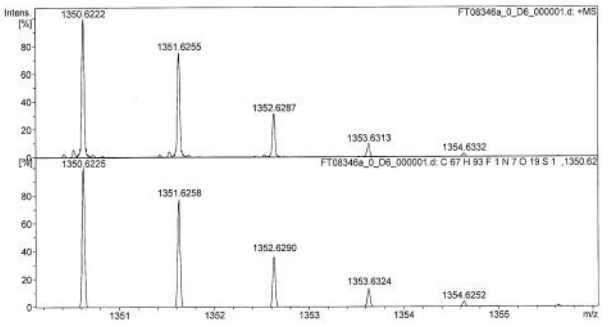
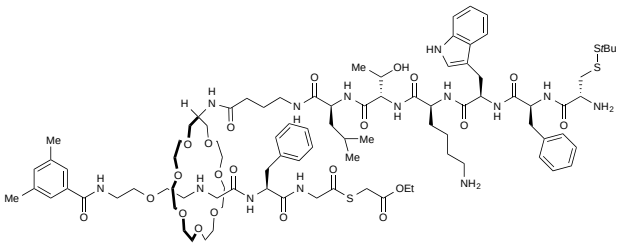


Fig. S25. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **S25**

Note: Products derived from [2]rotaxane S23 were obtained as a mixture of diastereomers. For simplicity, strucutres of one of the diastereomers are shown in the following sections.

7.5. Synthesis of lasso peptide L2

Peptido[2]rotaxane S26



S26

To a solution of rotaxane **S23** (16 mg, 12 μmol, 1.0 equiv) in DMSO (95 μL) was added Et_2NH (5 μL), and the mixture was incubated at RT for 4 min and directly added to a solution of peptide α-ketoacid **7** (13 mg, 14 μmol, 1.2 equiv) in DMSO/H_2O (6:4, 360 μL, 0.1 M oxalic acid). The resulting

mixture was incubated at 60 °C for 16 h and cooled to RT. Purification was performed by preparative HPLC using YMC C18 column (20 x 250 mm) with a gradient of 20 to 95% CH_3CN with 0.1% TFA in 28 min. The pure product fractions were pooled and lyophilized to obtain 11 mg of **S26** (51% yield for deprotection and KAHA ligation steps). Analytical HPLC and HRMS were used to confirm the purity and exact mass of the product.

HRMS (MALDI) calcd for $C_{92}H_{140}N_{14}NaO_{22}S_3$ $[M+Na]^+$: 1911.9321, found: 1911.9320.

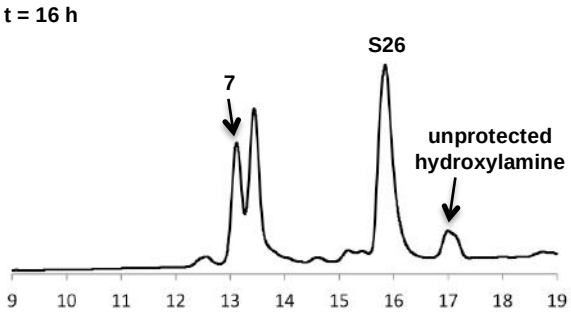


Fig. S26. HPLC monitoring of the KAHA ligation to form **S26**

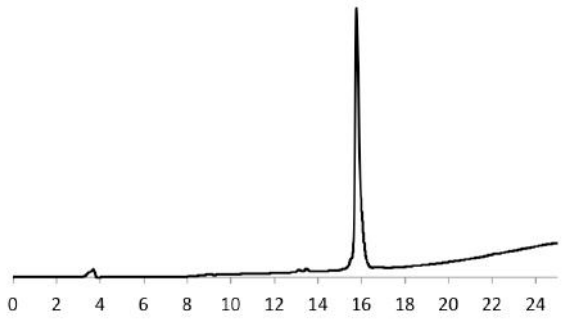


Fig. S27. Analytical HPLC of purified **S26**

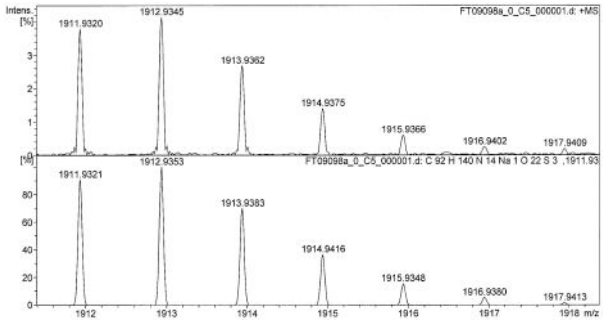
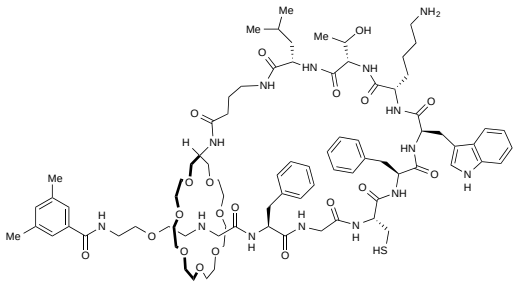


Fig. S28. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **S26**

Lasso peptide S27



S27

4-Mercaptophenylacetic acid (14 mg, 81 μmol, 10 equiv) and TCEP-HCl (46 mg, 162 μmol, 20 equiv) were dissolved in 0.25 M sodium phosphate buffer (pH 8.0)/CH_3CN (1:1, 2.1 mL), and pH of this solution was adjusted to 7.4 by adding 1 M aq NaOH. A solution of **S26** (15 mg, 8.1 μmol, 1.0 equiv) in 0.1 M sodium phosphate buffer (pH 7.4)/CH_3CN (1:1, 0.40 mL) was prepared, and a portion (100 μL) of this solution was added to the ligation buffer. The mixture was incubated at RT

for 15 min, and the next portion (100 μL) of the solution of **S26** was added to the mixture. This addition-incubation process was repeated every 15 minutes. After complete addition, the mixture was further incubated at RT for 1 h and purified by preparative HPLC using YMC C18 column (20 x 250 mm) with a gradient of 20 to 95% CH_3CN with 0.1% TFA in 28 min. The pure product fractions were pooled and lyophilized to obtain 5.3 mg of **S27** (39% yield). Analytical HPLC and HRMS were used to confirm the purity and exact mass of the product.

HRMS (MALDI) calcd for $C_{84}H_{125}N_{14}O_{20}S$ $[M+H]^+$: 1681.8910, found: 1681.8904.

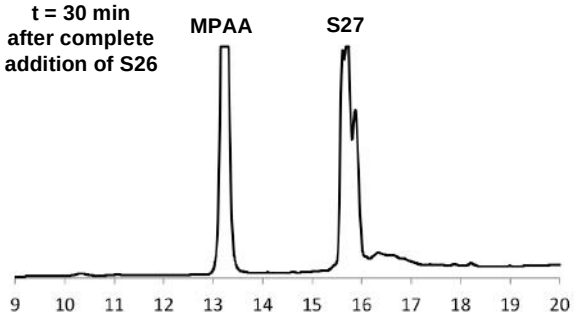


Fig. S29. HPLC monitoring of the NCL to form **S27**

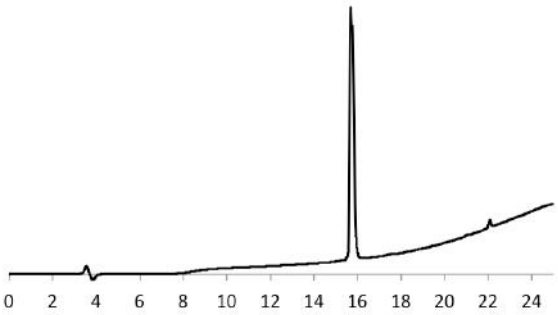


Fig. S30. Analytical HPLC of purified **S27**

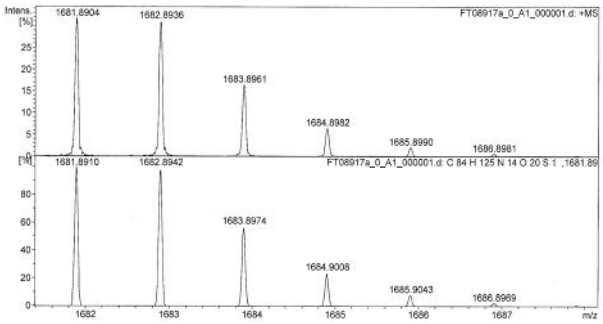
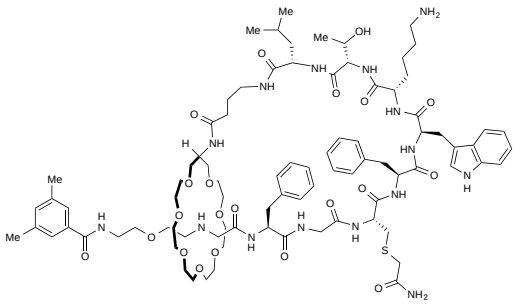


Fig. S31. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **S27**

Cys-alkylated lasso peptide L2



L2

To a solution of **S27** (5.3 mg, 3.2 μmol, 1.0 equiv) in 0.1 M sodium phosphate buffer (pH 7.4)/CH_3CN (1:1, 255 μL) was added a solution of iodoacetamide (0.65 mg, 3.5 μmol, 1.1 equiv) in 0.1 M sodium phosphate buffer (pH 7.4)/CH_3CN (1:1, 65 μL). The mixture was incubated at RT for 40 min and purified by preparative HPLC using YMC C18 column (20 x 250 mm) with a gradient of

20 to 95% CH_3CN with 0.1% TFA in 28 min. The pure product fractions were pooled and lyophilized to obtain 3.7 mg of **L2** (66% yield). Analytical HPLC and HRMS were used to confirm the purity and exact mass of the product.

HRMS (MALDI) calcd for $C_{86}H_{128}N_{15}O_{21}S$ $[M+H]^+$: 1738.9124, found: 1738.9098.

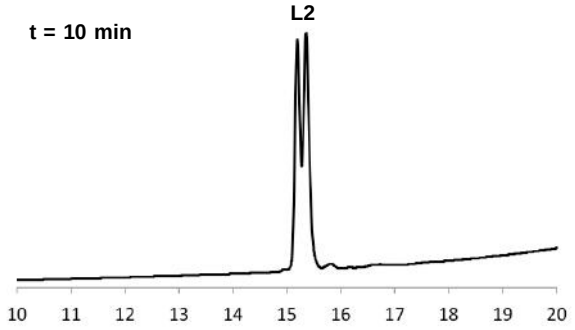


Fig. S32. HPLC monitoring of the cysteine alkylation to form **L2**

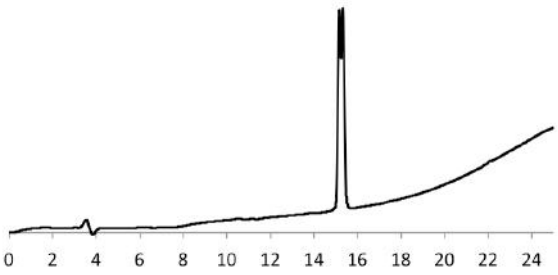


Fig. S33. Analytical HPLC of purified **L2**

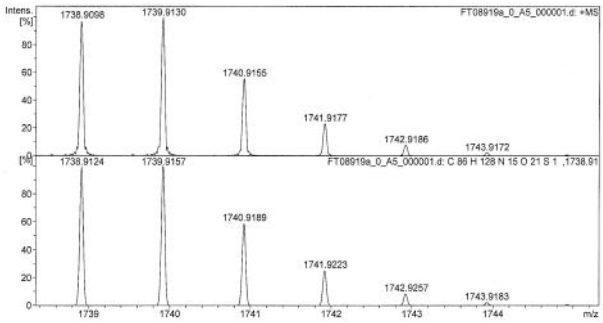
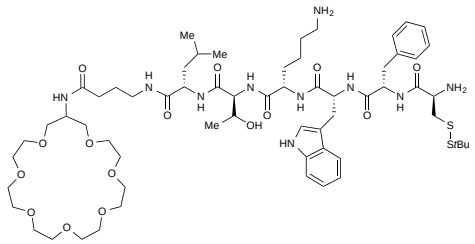


Fig. S34. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **L2**

8. Synthesis of branched-cyclic peptide B2

KAHA ligation product S28



S28

To a solution of crown ether **13** (16 mg, 21 μmol, 2.0 equiv) in DMSO (76 μL) was added Et_2NH (4.0 μL), and the mixture was incubated at RT for 4 min and directly added to a solution of peptide α-ketoacid **7** (9.6 mg, 11 μmol, 1.0 equiv) in DMSO/H_2O (6:4, 220 μL, 0.1 M oxalic acid). The resulting mixture was incubated at 60 °C for 20 h and cooled to RT. Purification was performed by preparative HPLC using YMC C18 column (20 x 250 mm) with a gradient of 20 to 95% CH_3CN with 0.1% TFA in 28 min. The pure product fractions were pooled and lyophilized to obtain 5.6 mg of **S28** (41% yield for deprotection and KAHA ligation steps). Analytical HPLC and HRMS were used

to confirm the purity and exact mass of the product.

HRMS (MALDI) calcd for $C_{62}H_{101}N_{10}O_{15}S_2$ $[M+H]^+$: 1289.6884, found: 1289.6881.

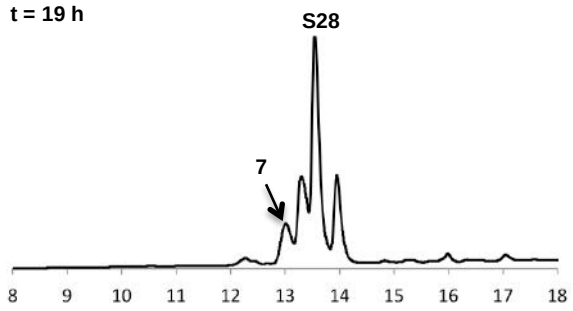


Fig. S35. HPLC monitoring of the KAHA ligation to form **S28**

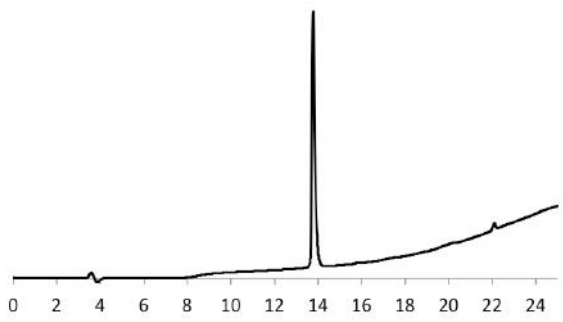


Fig. S36. Analytical HPLC of purified **S28**

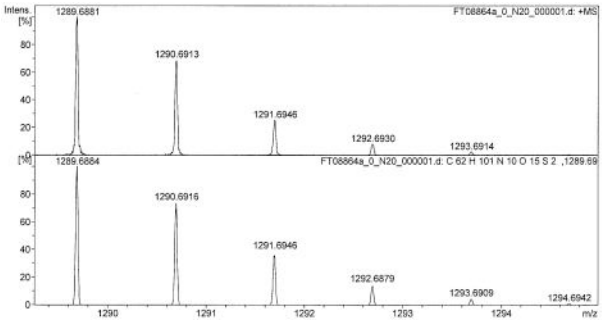
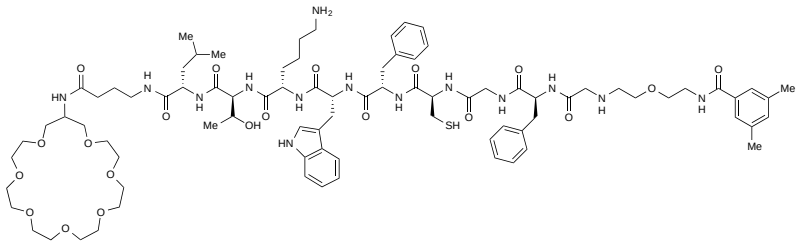


Fig. S37. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **S28**

NCL product S29



S29

4-Mercaptophenylacetic acid (7.3 mg, 43 μmol, 10 equiv) and TCEP-HCl (24.9 mg, 68 μmol, 20 equiv) were dissolved in 0.25 M sodium phosphate buffer (pH 8.0)/CH_3CN (1:1, 0.60 mL), and pH of this solution was adjusted to 7.4 by adding 1 M aq NaOH. To this ligation buffer were added a solution of **S28** (5.6 mg, 4.3 μmol, 1.0 equiv) in 0.1 M sodium phosphate buffer (pH 7.4)/CH_3CN (1:1, 0.40 mL) followed by a solution of axle-thioester **S24** (2.9 mg, 4.7 μmol, 1.1 equiv) in CH_3CN (0.10 mL), and the mixture was incubated at RT for 1.5 h. Purification was performed by preparative HPLC using YMC C18 column (20 x 250 mm) with a gradient of 20 to 95% CH_3CN with 0.1% TFA in 28 min. The pure product fractions were pooled and lyophilized to obtain 4.5 mg of **S29** (63% yield). Analytical HPLC and HRMS were used to confirm the purity and exact mass of the product.

HRMS (MALDI) calcd for $C_{84}H_{125}N_{14}O_{20}S$ $[M+H]^+$: 1681.8910, found: 1681.8901.

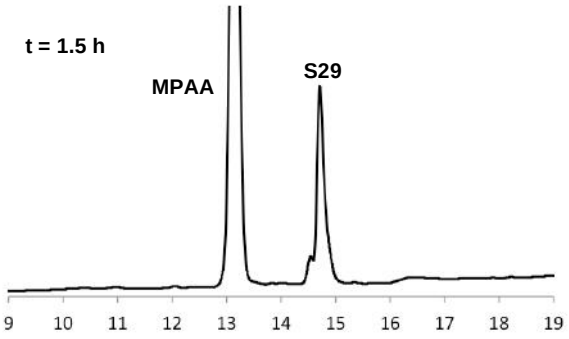


Fig. S38. HPLC monitoring of the NCL to form **S29**

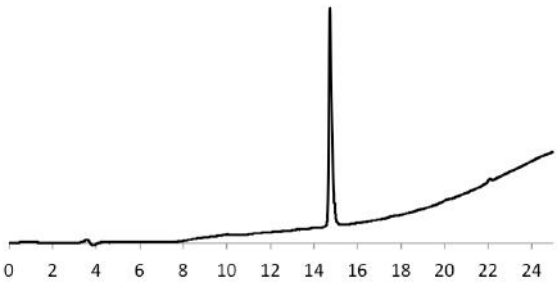


Fig. S39. Analytical HPLC of purified **S29**

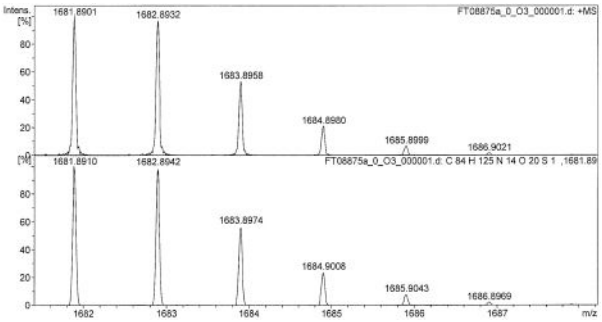
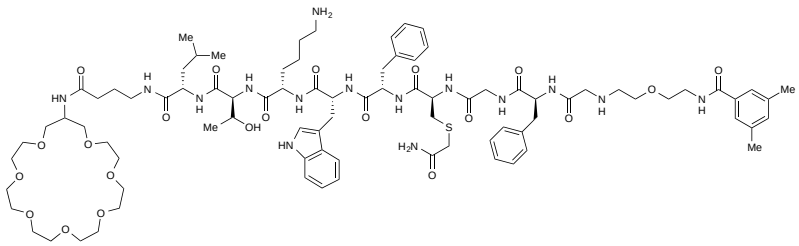


Fig. S40. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **S29**

Branched-cyclic peptide B2



B2

To a solution of **S29** (4.5 mg, 2.7 μmol, 1.0 equiv) in 0.1 M sodium phosphate buffer (pH 7.4)/CH_3CN (1:1, 211 μL) was added a solution of iodoacetamide (0.59 mg, 3.0 μmol, 1.2 equiv) in CH_3CN (59 μL). The mixture was incubated at RT for 1.5 h and purified by preparative HPLC using YMC C18 column (20 x 250 mm) with a gradient of 20 to 95% CH_3CN with 0.1% TFA in 28 min. The pure product fractions were pooled and lyophilized to obtain 3.3 mg of **B2** (70% yield). Analytical HPLC and HRMS were used to confirm the purity and exact mass of the product.

HRMS (MALDI) calcd for $C_{86}H_{128}N_{15}O_{21}S$ $[M+H]^+$: 1738.9124, found: 1738.9110.

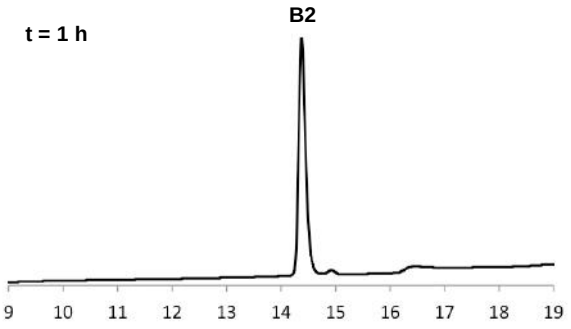


Fig. S41. HPLC monitoring of the cysteine alkylation to form **B2**

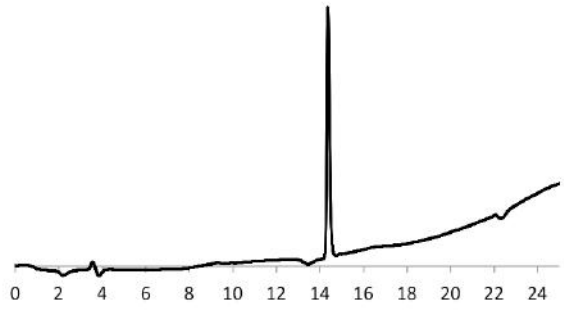


Fig. S42. Analytical HPLC of purified **B2**

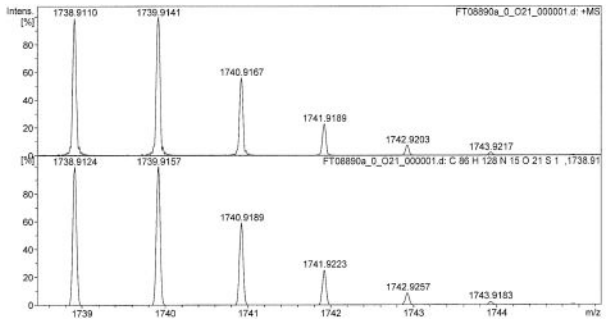
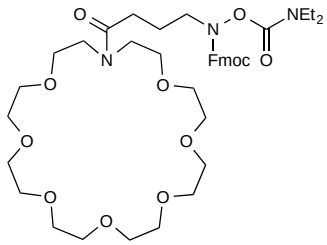


Fig. S43. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **B2**

9. Synthesis of lasso peptide L3

9.1. Synthesis of [2]rotaxane S30

Crown ether-*N*-Fmoc hydroxylamine 14



14

S14 (0.61 g, 1.4 mmol, 1.1 equiv), HCTU (0.57 g, 1.4 mmol, 1.1 equiv), and NMM (275 μL, 2.5 mmol, 2.0 equiv) were premixed in DMF (1.5 mL). 1-Aza-24-crown-8[13] (0.44 g, 1.3 mmol, 1.0 equiv) in DMF (1.0 mL) was added, and the mixture was stirred at RT for 1h. The reaction was diluted with DCM, and 1 M aq HCl was added. The aqueous phase was extracted with DCM (3x). The combined organic phases were washed with H_2O, dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was purified by preparative RP-HPLC using YMC C18 column (20 x 250 mm) with a gradient of 20 to 95% CH_3CN with 0.1% TFA in 28 min. The pure product fractions were pooled and lyophilized to obtain 0.29 g of **14** (30% yield) as a pale yellow oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.74 (d, *J* = 7.5 Hz, 2H), 7.58 (d, *J* = 7.4 Hz, 2H), 7.38 (t, *J* = 7.4 Hz,

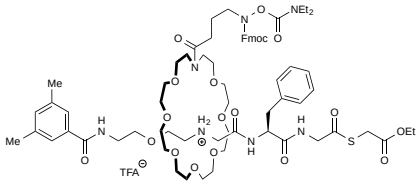
2H), 7.29 (td, *J* = 7.4 Hz, 2H), 4.45 (d, *J* = 7.2 Hz, 2H), 4.23 (t, *J* = 7.0 Hz, 1H), 3.73 (t, *J* = 6.9 Hz, 2H), 3.66-3.55 (m, 32H), 3.32 (t, *J* = 7.5 Hz, 4H), 2.50 (t, *J* = 7.5 Hz, 2H), 1.95 (p, *J* = 7.1 Hz, 2H), 1.17 (t, *J* = 7.1 Hz, 6H).

^{13}C NMR (100 MHz, $CDCl_3$) δ 173.52, 155.96, 153.95, 143.63, 141.22, 127.69, 127.05, 125.04, 119.89, 70.80, 70.69, 70.66, 70.64, 70.58, 70.48, 70.43, 69.62, 69.38, 68.20, 50.08, 49.21, 47.12, 46.99, 42.99, 41.55, 29.92, 22.77, 14.04, 13.26.

IR (thin film) 2935, 2873, 1744, 1642, 1451, 1421, 1350, 1144, 1111 cm^{-1}.

HRMS (ESI) calcd for $C_{40}H_{60}N_3O_{12}$ $[M+H]^+$: 774.4172, found: 774.4157.

[2]Rotaxane S30



S30

3,5-Dimethylphenyl KAT **15** (98 mg, 0.41 mmol, 1.1 equiv), crown ether **14** (0.29 g, 0.37 mmol, 1.0 equiv), and **3** (0.24 g, 0.37 mmol, 1.0 equiv) were dissolved in CH_3CN (1.9 mL), and HBF_4•OEt_2 (104 μL, 0.82 mmol, 2.0 equiv) was added. The mixture was stirred at RT for 16 h. The reaction was quenched with H_2O. The crude material was purified by preparative HPLC using a YMC C18 column with a gradient of 40 to 95% CH_3CN with 0.1% TFA in 38 min. The pure product fractions were pooled and lyophilized to obtain 63 mg of **S30** (12% yield).

In the same purification, the peaks containing axle **S24** were also collected, and these fractions were pooled and lyophilized. The lyophilizates were repurified by preparative HPLC using a YMC C18 column with a gradient of 20 to 95% CH_3CN with 0.1% TFA in 28 min. The pure product fractions were pooled and lyophilized to obtain 93 mg of **S24** (36% yield).

^{1}H NMR (600 MHz, $CDCl_3$) δ 9.53 (dd, *J* = 22.9, 8.1 Hz, 1H), 8.77-8.73 (m, 1H), 7.73 (d, *J* = 7.6 Hz, 2H), 7.63-7.59 (m, 1H), 7.57-7.56 (m, 2H), 7.52-7.47 (m, 1H), 7.37 (t, *J* = 7.5 Hz, 2H), 7.33-7.26 (m, 6H), 7.21-7.18 (m, 2H), 7.14-7.10 (m, 1H), 7.09-7.06 (m, 2H), 4.85-4.79 (m, 1H), 4.42 (d, *J* = 7.0 Hz, 2H), 4.21 (t, *J* = 7.1 Hz, 1H), 4.14-4.10 (m, 4H), 3.99-3.83 (m, 2H), 3.75-3.29 (m, 49H), 2.98-2.93 (m, 1H), 2.39-2.32 (m, 2H), 2.30 (s, 6H), 1.95-1.90 (m, 2H), 1.23 (t, *J* = 7.1 Hz, 3H), 1.18-1.14 (m, 6H).

^{13}C NMR (150 MHz, $CDCl_3$) δ 195.81, 195.79, 172.69, 172.65, 172.01, 171.97, 168.54, 168.43, 165.23, 165.21, 161.01 (q, *J* = 36.5 Hz), 156.02, 153.89, 143.55, 143.54, 141.16, 138.05, 137.67, 137.63, 134.56, 134.55, 133.00, 129.26, 129.22, 128.20, 127.70, 127.02, 126.33, 126.30, 125.00, 124.69, 119.88, 116.23 (q, *J* = 292 Hz), 71.32, 71.27, 71.06, 70.97, 70.95, 70.83, 70.80, 70.76, 70.74, 70.72, 70.68, 70.65, 70.62, 70.56, 70.51, 70.49, 70.47, 70.45, 70.42, 70.40, 69.78, 68.17, 65.76, 61.66, 55.95, 55.76, 50.04, 49.15, 48.92, 48.22, 48.18, 47.98, 47.95, 46.93, 46.63, 46.58,

42.96, 41.52, 39.40, 37.96, 37.94, 30.80, 29.67, 29.65, 22.53, 21.13, 14.05, 13.97, 13.25.

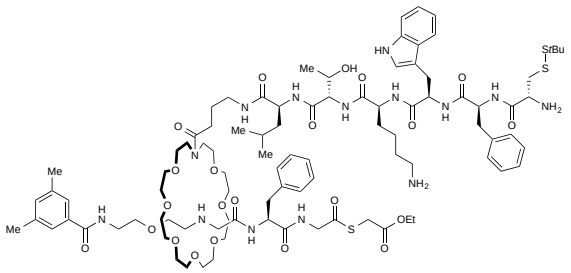
$[\alpha]_D^{26}$ = –5.8° (c 0.70, $CHCl_3$)

IR (thin film) 2910, 1741, 1690, 1647, 1537, 1451, 1421, 1200, 1093 cm^{-1}.

HRMS (MALDI) calcd for $C_{70}H_{100}N_7O_{19}S$ $[M+H–TFA]^+$: 1374.6789, found: 1374.6753.

9.2. Synthesis of lasso peptide L3

Peptido[2]rotaxane S31



S31

To a solution of rotaxane **S30** (35 mg, 24 μmol, 1.0 equiv) in DMSO (190 μL) was added Et_2NH (10 μL), and the mixture was incubated at RT for 4 min and directly added to a solution of peptide α-ketoacid **7** (27 mg, 30 μmol, 1.3 equiv) in DMSO/H_2O (6:4, 400 μL, 0.1 M oxalic acid). The resulting mixture was incubated at 60 °C for 15 h and cooled to RT. Purification was performed by preparative HPLC using YMC C18 column (20 x 250 mm) with a gradient of 20 to 95% CH_3CN with 0.1% TFA in 28 min. The pure product fractions were pooled and lyophilized to obtain 13 mg of **S31** (29% yield for deprotection and KAHA ligation steps). Analytical HPLC and HRMS were used to confirm the purity and exact mass of the product.

HRMS (MALDI) calcd for $C_{93}H_{143}N_{14}O_{22}S_3$ $[M+H]^+$: 1903.9658, found: 1903.9656.

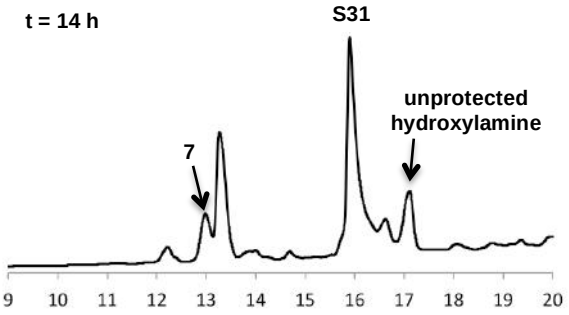


Fig. S44. HPLC monitoring of the KAHA ligation to form **S31**

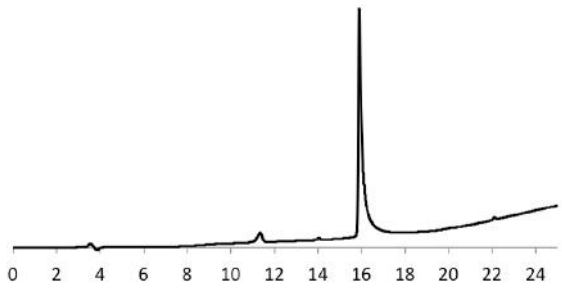


Fig. S45. Analytical HPLC of purified **S31**

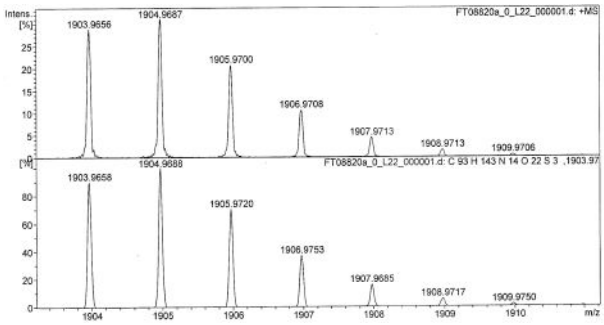
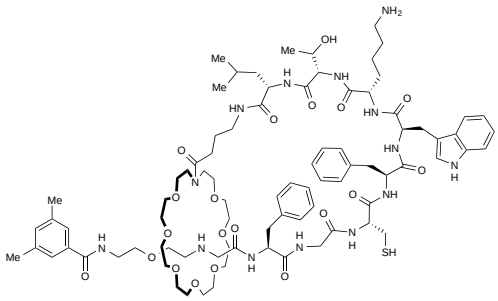


Fig. S46. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **S31**

Lasso peptide S32



S32

4-Mercaptophenylacetic acid (11 mg, 63 μmol, 10 equiv) and TCEP-HCl (36 mg, 126 μmol, 20 equiv) were dissolved in 0.25 M sodium phosphate buffer (pH 8.0)/CH_3CN (1:1, 0.63 mL), and pH of this solution was adjusted to 7.5 by adding 1 M aq NaOH. A solution of **S31** (12 mg, 6.3 μmol, 1.0 equiv) in 0.1 M sodium phosphate buffer (pH 7.4)/CH_3CN (1:1, 0.20 mL) was prepared, and a portion (20 μL) of this solution was added to the ligation buffer. The mixture was incubated at RT for 5 min, and the next portion (20 μL) of the solution of **S31** was added to the mixture. This addition-incubation process was repeated every 5 minutes. After complete addition, the mixture was further incubated at RT for 30 min and purified by preparative HPLC using YMC C18 column (20 x 250 mm) with a gradient of 20 to 95% CH_3CN with 0.1% TFA in 28 min. The pure product fractions were pooled and lyophilized to obtain 5.2 mg of **S32** (49% yield). Analytical HPLC and HRMS were used to confirm the purity and exact mass of the product.

HRMS (MALDI) calcd for $C_{85}H_{127}N_{14}O_{20}S$ $[M+H]^+$: 1695.9066, found: 1695.9068.

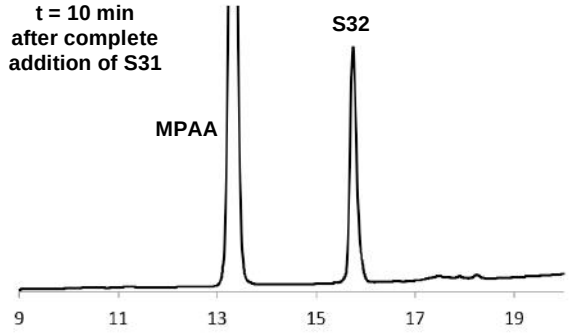


Fig. S47. HPLC monitoring of the NCL to form **S32**

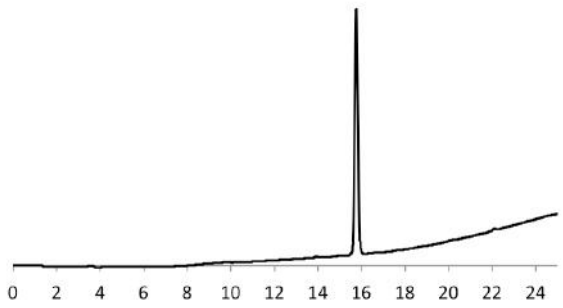


Fig. S48. Analytical HPLC of purified **S32**

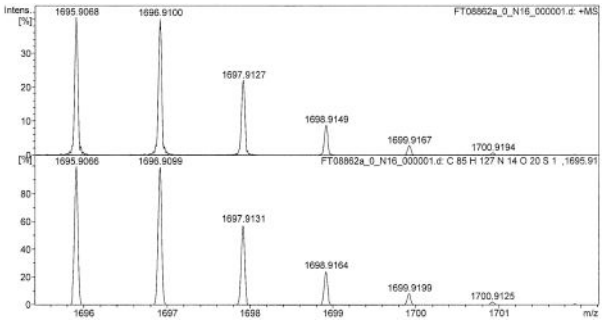
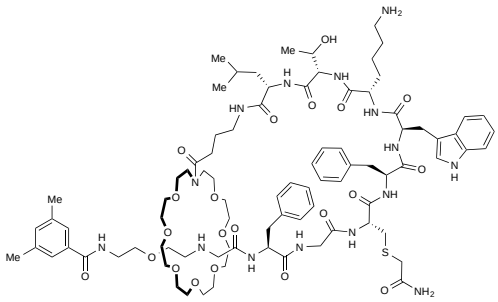


Fig. S49. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **S32**

Cys-alkylated lasso peptide L3



L3

To a solution of **S32** (7.1 mg, 4.2 μmol, 1.0 equiv) in 0.1 M sodium phosphate buffer (pH 7.4)/CH_3CN (1:1, 327 μL) was added a solution of iodoacetamide (0.93 mg, 5.0 μmol, 1.2 equiv) in CH_3CN (59 μL). The mixture was incubated at RT for 40 min and purified by preparative HPLC using YMC C18 column (20 x 250 mm) with a gradient of 20 to 95% CH_3CN with 0.1% TFA in 28 min. The pure product fractions were pooled and lyophilized to obtain 4.4 mg of **L3** (60% yield). Analytical HPLC and HRMS were used to confirm the purity and exact mass of the product.

HRMS (MALDI) calcd for $C_{87}H_{130}N_{15}O_{21}S$ $[M+H]^+$: 1752.9281, found: 1752.9265.

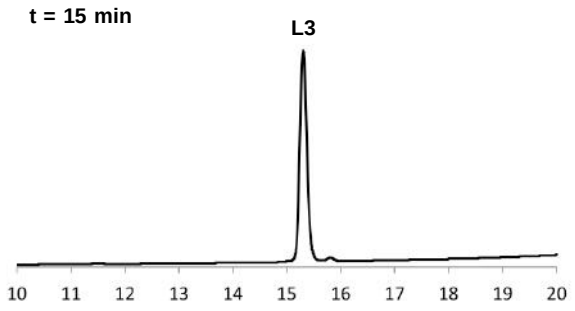


Fig. S50. HPLC monitoring of the cysteine alkylation to form **L3**

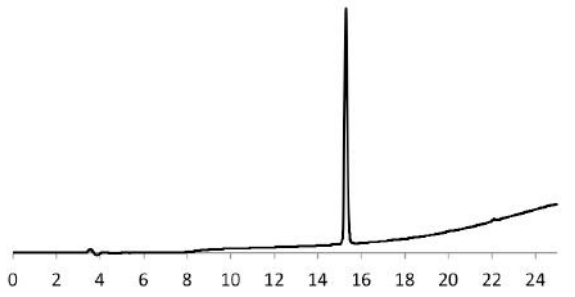


Fig. S51. Analytical HPLC of purified **L3**

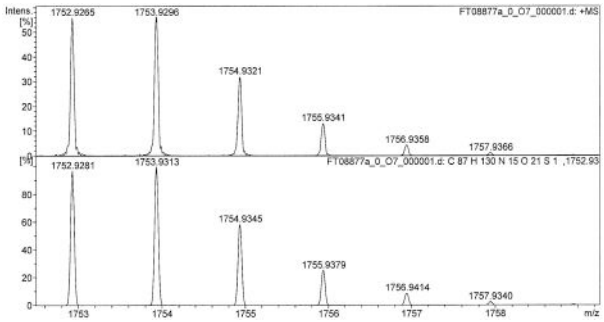
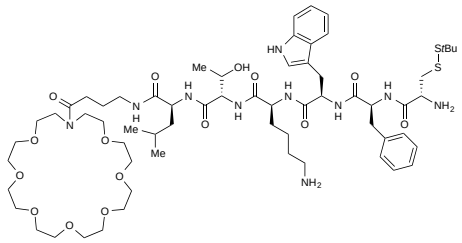


Fig. S52. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **L3**

10. Synthesis of branched-cyclic peptide B3

KAHA ligation product S33



S33

To a solution of crown ether **14** (7.8 mg, 10 μmol, 1.0 equiv) in DMSO (57 μL) was added Et_2NH (3.0 μL), and the mixture was incubated at RT for 4 min and directly added to a solution of peptide α-ketoacid **7** (11 mg, 12 μmol, 1.2 equiv) in DMSO/H_2O (6:4, 240 μL, 0.1 M oxalic acid). The resulting mixture was incubated at 60 °C for 19 h and cooled to RT. Purification was performed by preparative HPLC using YMC C18 column (20 x 250 mm) with a gradient of 20 to 95% CH_3CN with 0.1% TFA in 28 min. The pure product fractions were pooled and lyophilized to obtain 4.2 mg of **S33** (32% yield for deprotection and KAHA ligation steps). Analytical HPLC and HRMS were used to confirm the purity and exact mass of the product.

HRMS (MALDI) calcd for $C_{63}H_{103}N_{10}O_{15}S_2$ $[M+H]^+$: 1303.7040, found: 1303.7036.

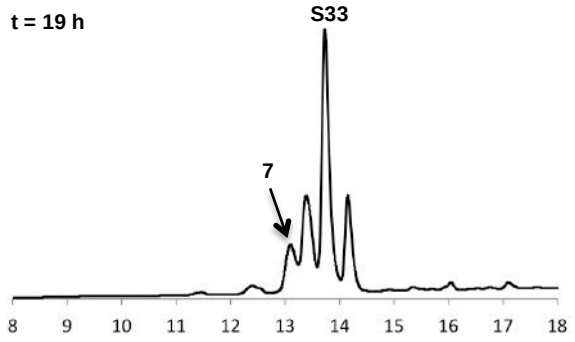


Fig. S53. HPLC monitoring of the KAHA ligation to form **S33**

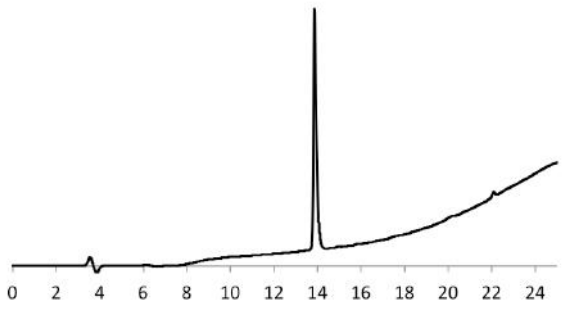


Fig. S54. Analytical HPLC of purified **S33**

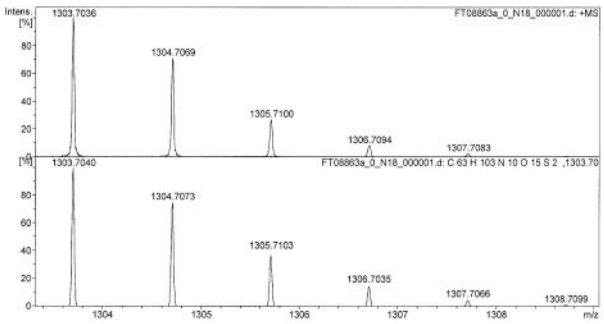
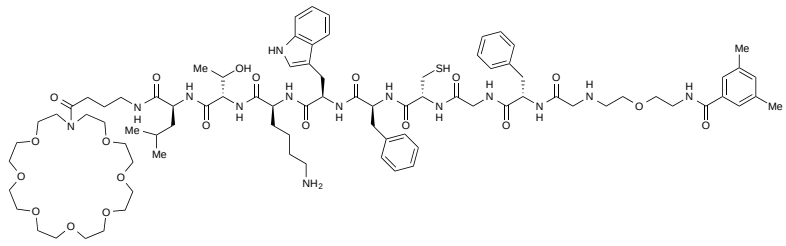


Fig. S55. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **S33**

NCL product S34



S34

4-Mercaptophenylacetic acid (5.4 mg, 32 μmol, 10 equiv) and TCEP-HCl (18 mg, 64 μmol, 20 equiv) were dissolved in 0.25 M sodium phosphate buffer (pH 8.0)/CH_3CN (1:1, 0.50 mL), and pH of this solution was adjusted to 7.2 by adding 1 M aq NaOH. To this ligation buffer were added a solution of **S33** (4.2 mg, 3.2 μmol, 1.0 equiv) in 0.1 M sodium phosphate buffer (pH 7.4)/CH_3CN (1:1, 0.30 mL) followed by a solution of axle-thioester **S24** (2.1 mg, 3.5 μmol, 1.1 equiv) in CH_3CN (0.10 mL), and the mixture was incubated at RT for 1 h. Purification was performed by preparative HPLC using YMC C18 column (20 x 250 mm) with a gradient of 20 to 95% CH_3CN with 0.1% TFA in 28 min. The pure product fractions were pooled and lyophilized to obtain 3.2 mg of **S34** (59% yield). Analytical HPLC and HRMS were used to confirm the purity and exact mass of the product.

HRMS (MALDI) calcd for $C_{85}H_{127}N_{14}O_{20}S$ $[M+H]^+$: 1695.9066, found: 1695.9051.

S34

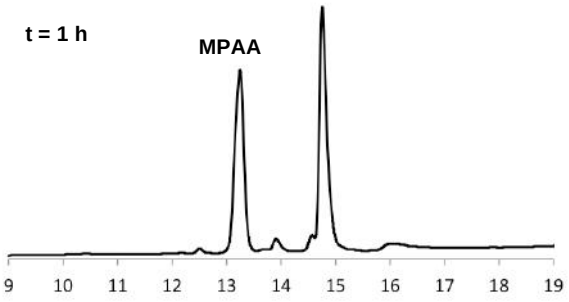


Fig. S56. HPLC monitoring of the NCL to form **S34**

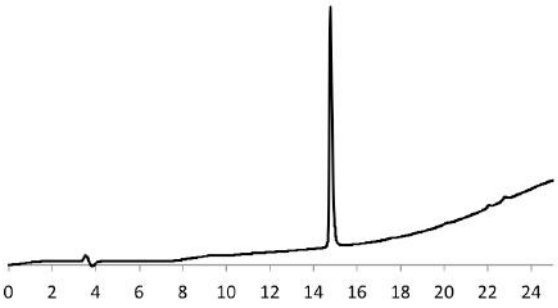


Fig. S57. Analytical HPLC of purified **S34**

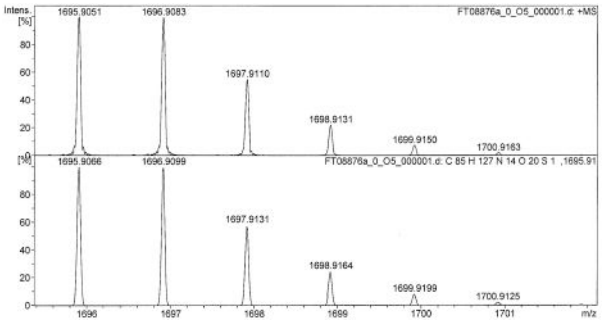
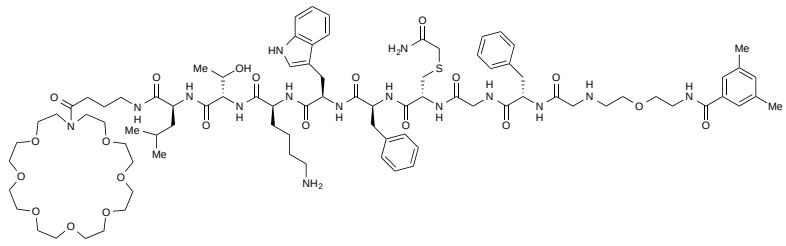


Fig. S58. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **S34**

Branched-cyclic peptide B3



B3

To a solution of **S34** (3.2 mg, 1.9 μmol, 1.0 equiv) in 0.1 M sodium phosphate buffer (pH 7.4)/CH_3CN (1:1, 148 μL) was added a solution of iodoacetamide (0.42 mg, 2.3 μmol, 1.2 equiv) in CH_3CN (42 μL). The mixture was incubated at RT for 1.5 h and purified by preparative HPLC using YMC C18 column (20 x 250 mm) with a gradient of 20 to 95% CH_3CN with 0.1% TFA in 28 min. The pure product fractions were pooled and lyophilized to obtain 2.6 mg of **B3** (78% yield). Analytical HPLC and HRMS were used to confirm the purity and exact mass of the product.

HRMS (MALDI) calcd for $C_{87}H_{130}N_{15}O_{21}S$ $[M+H]^+$: 1752.9281, found: 1752.9272.

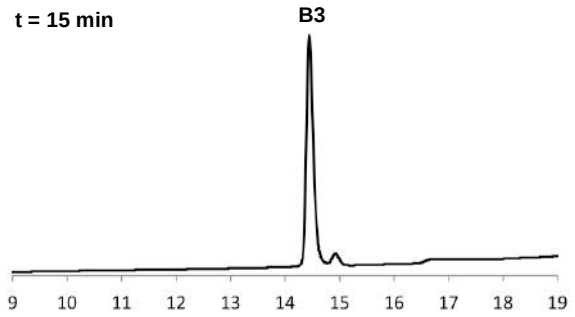


Fig. S59. HPLC monitoring of the cysteine alkylation to form **B3**

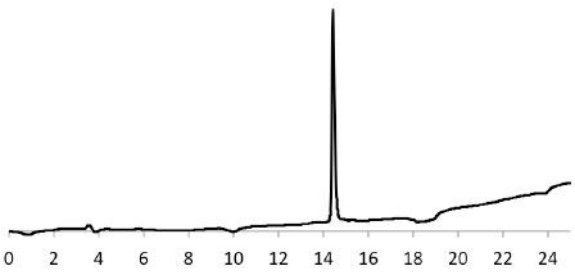


Fig. S60. Analytical HPLC of purified **B3**

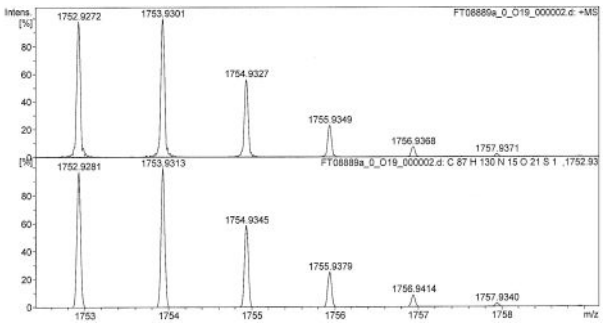


Fig. S61. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **B3**

11. Comparison of HPLC retention time

11.1. Lasso peptide L1 vs Branched-cyclic peptide B1

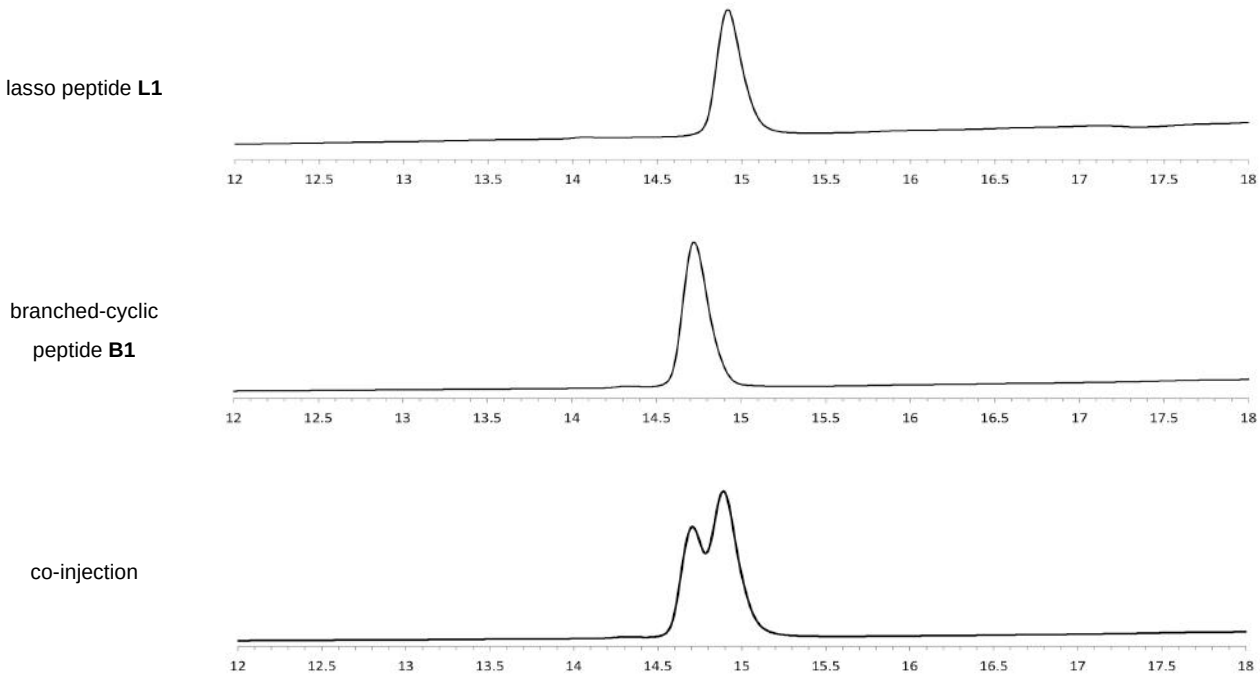


Fig. S62. Distinction between **L1** and **B1** by HPLC

11.2. Lasso peptide L2 vs Branched-cyclic peptide B2

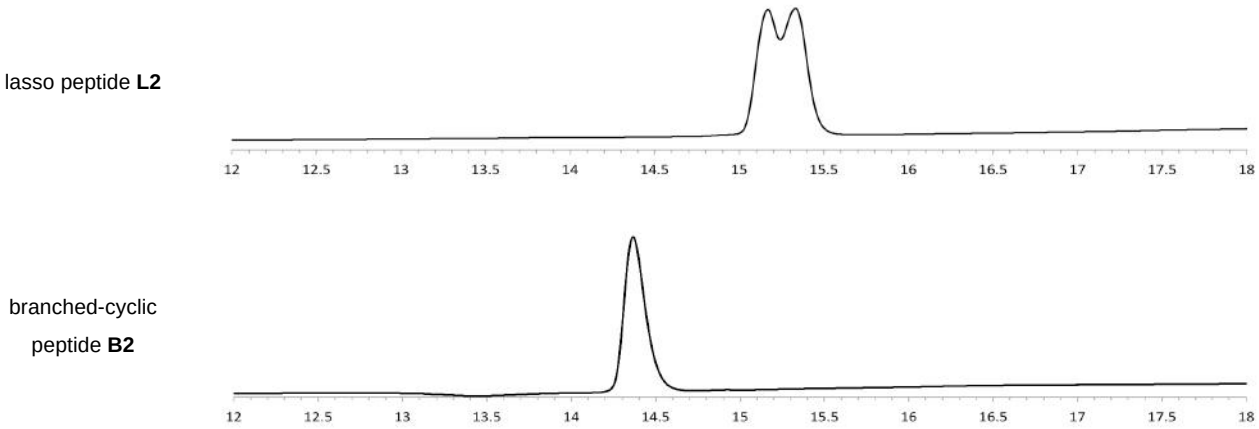


Fig. S63. Distinction between **L2** and **B2** by HPLC

11.3. Lasso peptide L3 vs Branched-cyclic peptide B3

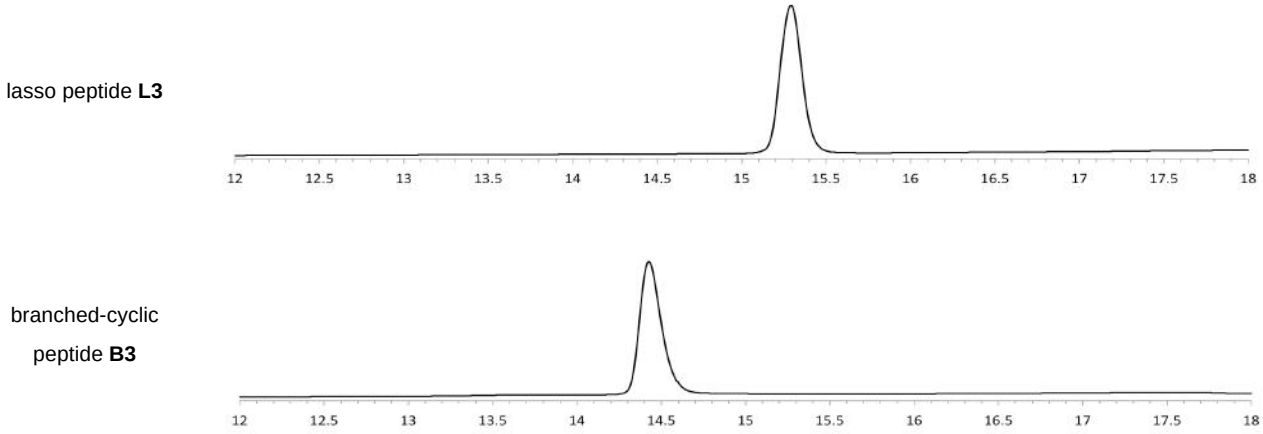


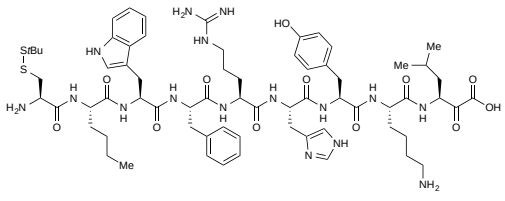
Fig. S64. Distinction between **L3** and **B3** by HPLC

12. Syntheses of lasso peptides with other peptide sequences

12.1. Peptide sequence from antibody Fc-region binder

12.1.1. Synthesis of lasso peptide 10

Peptide α-ketoacid S35



S35

Peptide α-ketoacid **S35** was prepared using the protected leucine α-ketoacid resin **S1** on a 0.24 mmol scale (1.4 g) with a substitution capacity of 0.17 mmol/g. After the full assembly of amino acids, the resin was treated with (95:2.5:2.5) TFA:DODT:H_2O for 1.5 h and removed by filtration. The volatiles were evaporated from the filtrate under reduced pressure. The residue was triturated

with MTBE and centrifuged to obtain the crude **S35**. Purification of crude **S35** was performed by preparative HPLC using YMC C18 column (20 x 250 mm) with a gradient of 20 to 95% CH_3CN with 0.1% TFA in 28 min. The pure product fractions were pooled and lyophilized to obtain 32 mg of **S35** (10% yield for peptide synthesis, resin cleavage and purification steps). Analytical HPLC and HRMS were used to confirm the purity and exact mass of the product.

HRMS (MALDI) calcd for $C_{67}H_{97}N_{16}O_{12}S_2$ $[M+H]^+$: 1381.6908, found: 1381.6892.

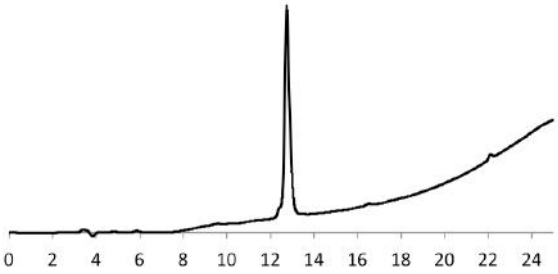


Fig. S65. Analytical HPLC of purified **S35**

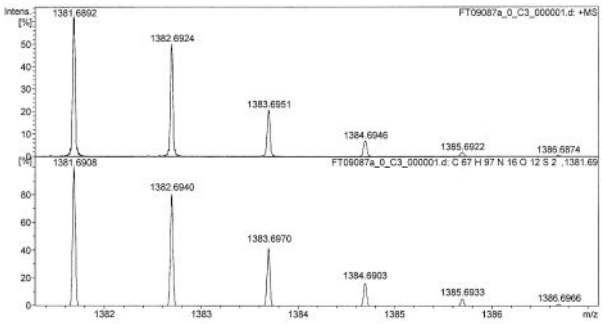
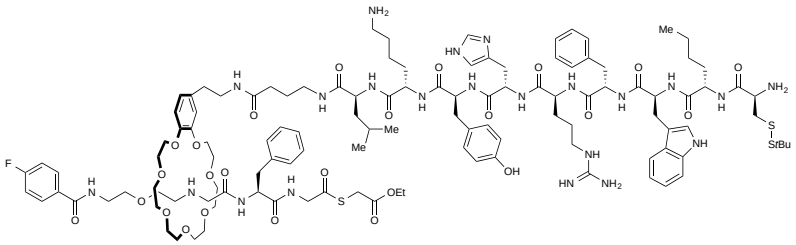


Fig. S66. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **S35**

Peptido[2]rotaxane S36



S36

To a solution of rotaxane **4** (13 mg, 9.2 μmol, 1.0 equiv) in DMSO (95 μL) was added Et_2NH (5 μL), and the mixture was incubated at RT for 4 min and directly added to a solution of peptide α-ketoacid **S35** (17 mg, 12 μmol, 1.3 equiv) in DMSO/H_2O (6:4, 268 μL, 0.1 M oxalic acid). The resulting mixture was incubated at 60 °C for 17 h and cooled to RT. Purification was performed by preparative HPLC using YMC C18 column (20 x 250 mm) with a gradient of 20 to 95% CH_3CN with 0.1% TFA in 28 min. The pure product fractions were pooled and lyophilized to obtain 4.8 mg of **S36** (22% yield for deprotection and KAHA ligation steps). Analytical HPLC and HRMS were used to confirm the purity and exact mass of the product.

HRMS (MALDI) calcd for $C_{118}H_{170}FN_{22}O_{25}S_3$ $[M+H]^+$: 2410.1848, found: 2410.1897.

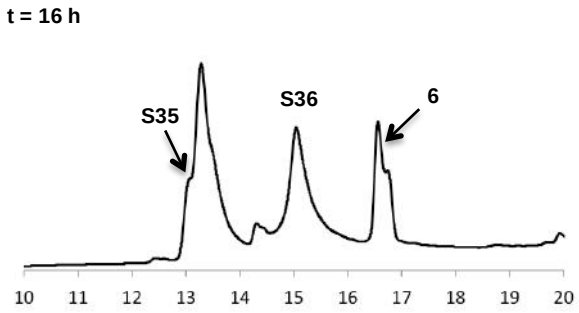


Fig. S67. HPLC monitoring of the KAHA ligation to form **S36**

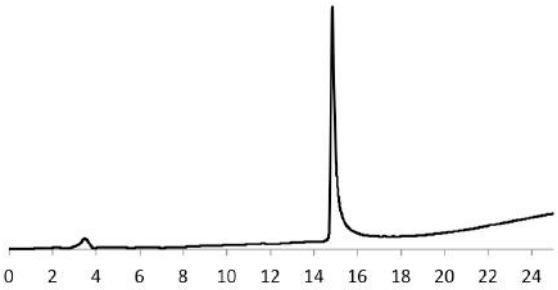


Fig. S68. Analytical HPLC of purified **S36**

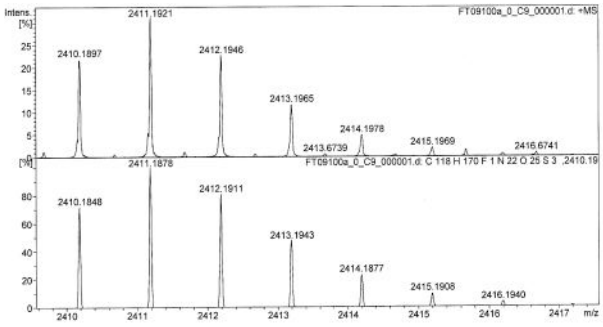
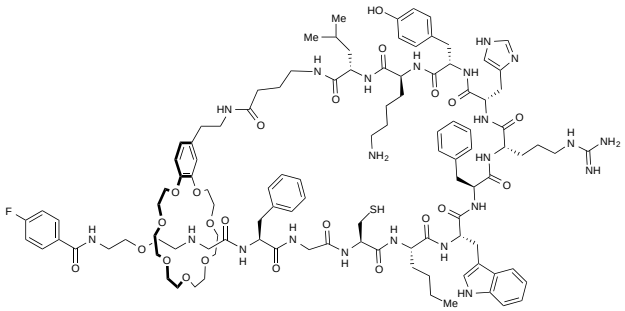


Fig. S69. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **S36**

Lasso peptide S37



S37

4-Mercaptophenylacetic acid (3.2 mg, 19 μmol, 10 equiv) and TCEP-HCl (11 mg, 38 μmol, 20 equiv) were dissolved in 0.25 M sodium phosphate buffer (pH 8.0)/CH_3CN (1:1, 475 μL), and pH of this solution was adjusted to 7.3 by adding 1 M aq NaOH. A solution of **S36** (4.6 mg, 1.9 μmol, 1.0 equiv) in 0.1 M sodium phosphate buffer (pH 7.4)/CH_3CN (1:1, 200 μL) was prepared, and a portion (50 μL) of this solution was added to the ligation buffer. The mixture was incubated at RT for 20 min, and the next portion (50 μL) of the solution of **S36** was added to the mixture. This addition-incubation process was repeated every 5 minutes. After complete addition, the mixture was further incubated at RT for 40 min and purified by preparative HPLC using YMC C18 column (20 x 250 mm) with a gradient of 20 to 95% CH_3CN with 0.1% TFA in 28 min. The pure product fractions were pooled and lyophilized to obtain 2.7 mg of **S37** (65% yield). Analytical HPLC and HRMS were used to confirm the purity and exact mass of the product.

HRMS (MALDI) calcd for $C_{110}H_{153}FN_{22}NaO_{23}S$ $[M+Na]^+$: 2224.1076, found: 2224.1118.

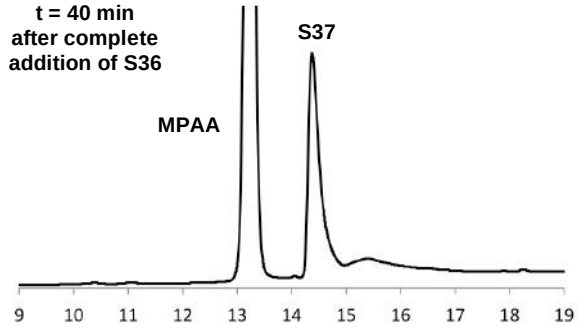


Fig. S70. HPLC monitoring of the NCL to form **S37**

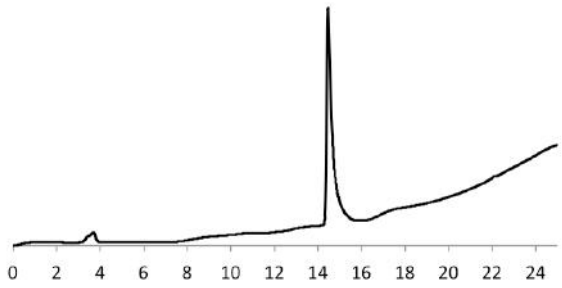


Fig. S71. Analytical HPLC of purified **S37**

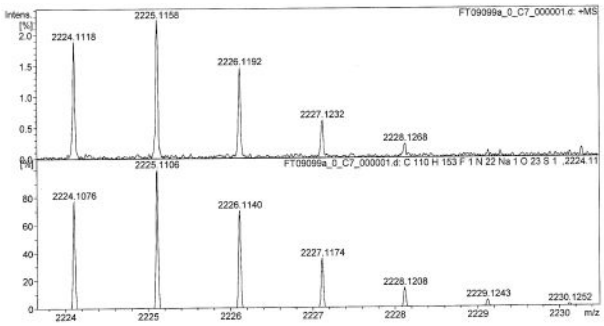
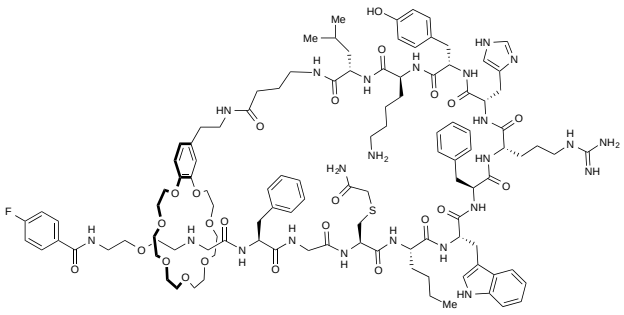


Fig. S72. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **S37**

Cys-alkylated lasso peptide 10



10

To a solution of **S37** (2.7 mg, 1.2 μmol, 1.0 equiv) in 0.1 M sodium phosphate buffer (pH 7.4)/CH_3CN (1:1, 240 μL) was added a solution of iodoacetamide (0.27 mg, 1.4 μmol, 1.2 equiv) in CH_3CN (27 μL). The mixture was incubated at RT for 40 min and purified by preparative HPLC using YMC C18 column (20 x 250 mm) with a gradient of 20 to 95% CH_3CN with 0.1% TFA in 28 min. The pure product fractions were pooled and lyophilized to obtain 1.0 mg of **10** (37% yield). Analytical HPLC and HRMS were used to confirm the purity and exact mass of the product.

HRMS (MALDI) calcd for $C_{112}H_{157}FN_{23}O_{24}S$ $[M+H]^+$: 2259.1471, found: 2259.1504.

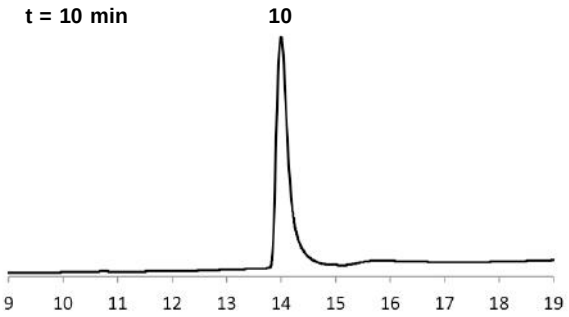


Fig. S73. HPLC monitoring of the cysteine alkylation to form **10**

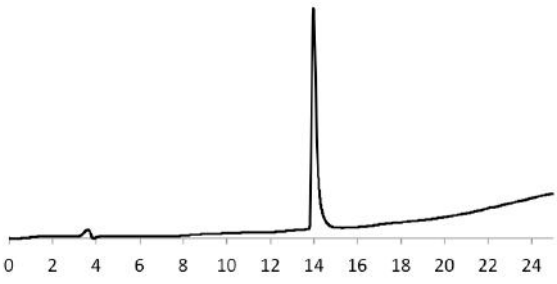


Fig. S74. Analytical HPLC of purified **10**

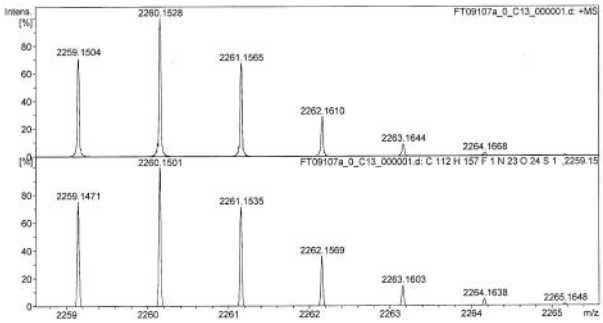


Fig. S75. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **10**

12.1.2. Trypsin digestion of 10

In order to briefly confirm the formation of **10**, trypsin digestion of **10** was conducted. A solution of **10** in H_2O (1 μg/μL) and a solution of trypsin in H_2O (1 μg/μL) were prepared. A solution of 20 μg of peptide was diluted with 50 μL of a buffer (50 mM Tris-HCl, 1 mM $CaCl_2$, pH 7.6). To this solution was added a solution of 1 μg of trypsin. The mixture was incubated at RT for 4 h. The major degradation product was analyzed and identified as [2]rotaxane **S38** by HRMS (MALDI) analysis.

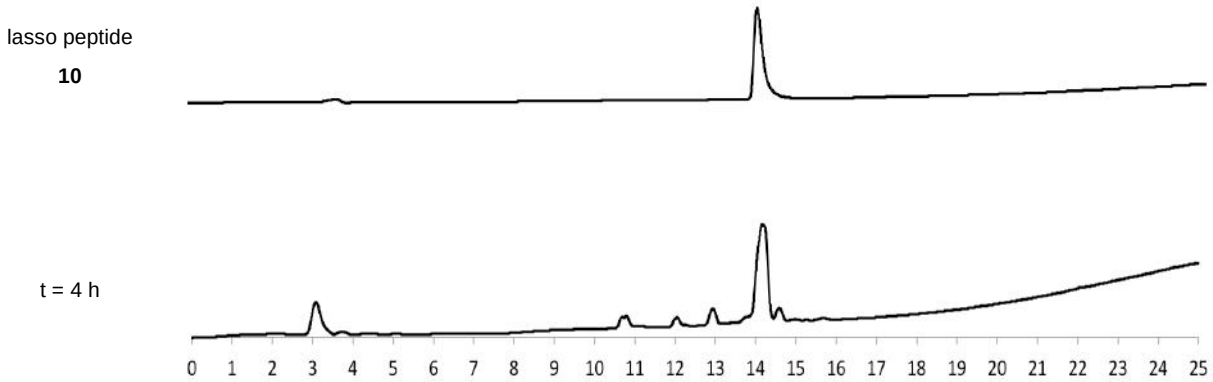
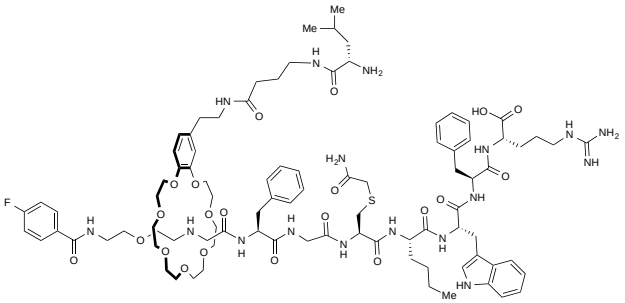


Fig. S76. Trypsin digestion of **10**

Degradation product **S38**



HRMS (MALDI) calcd for $C_{91}H_{131}FN_{17}O_{21}S$ $[M+H]^+$: 1848.9405, found: 1848.9399.

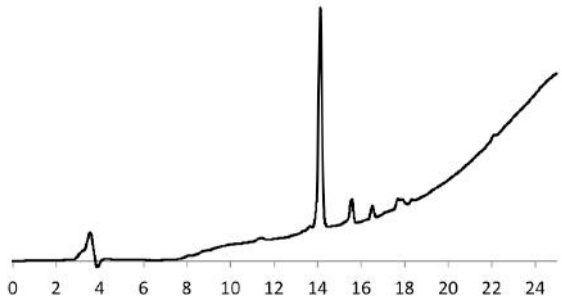


Fig. S77. Analytical HPLC of purified **S38**

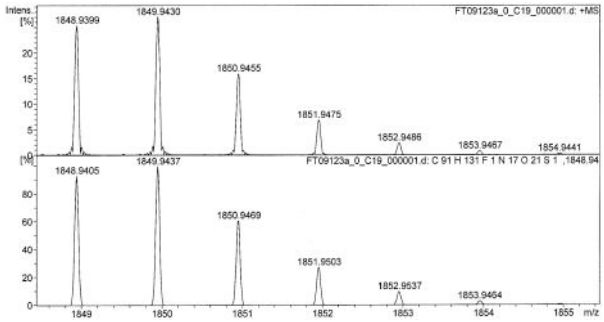
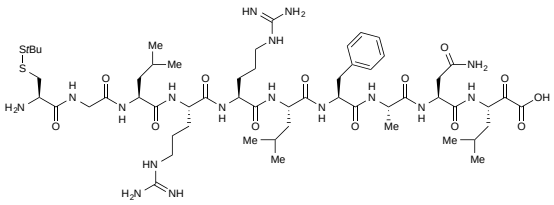


Fig. S78. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **S38**

12.2. Peptide sequence from lassomycin

12.2.1. Synthesis of lasso peptide 11

Peptide α-ketoacid S39



S39

Peptide α-ketoacid **S39** was prepared using the protected leucine α-ketoacid resin **S1** on a 0.10 mmol scale (0.49 g) with a substitution capacity of 0.20 mmol/g. After the full assembly of amino acids, the resin was treated with (95:2.5:2.5) TFA:DODT:H_2O for 1.5 h and removed by filtration. The volatiles were evaporated from the filtrate under reduced pressure. The residue was triturated with Et_2O and centrifuged to obtain the crude **S39**. Purification of crude **S39** was performed by preparative HPLC using YMC C18 column (20 x 250 mm) with a gradient of 20 to 95% CH_3CN with 0.1% TFA in 28 min. The pure product fractions were pooled and lyophilized to obtain 45 mg of **S39** (36% yield for peptide synthesis, resin cleavage and purification steps). Analytical HPLC and HRMS were used to confirm the purity and exact mass of the product.

HRMS (MALDI) calcd for $C_{56}H_{96}N_{17}O_{13}S_2$ $[M+H]^+$: 1278.6809, found: 1278.6816.

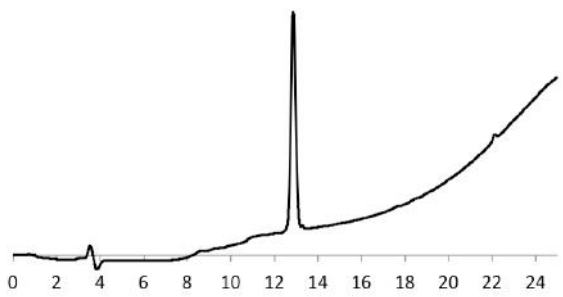


Fig. S79. Analytical HPLC of purified **S39**

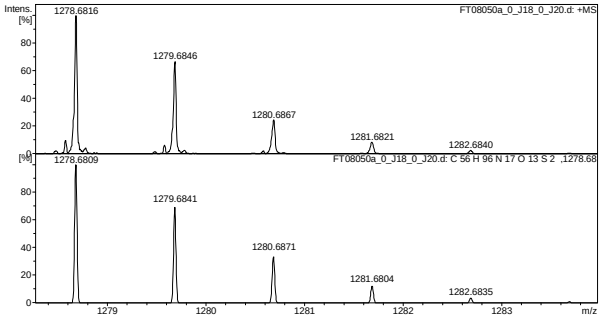
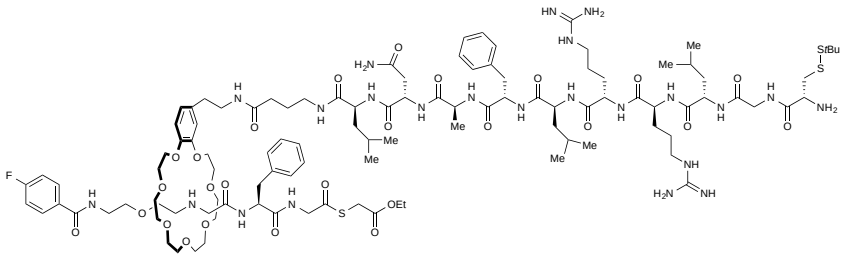


Fig. S80. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **S39**

Peptido[2]rotaxane S40



S40

Hydroxylamine **6** (21 mg, 18 μmol, 1.5 equiv) and α-ketoacid **S39** (15 mg, 12 μmol, 1.0 equiv) were dissolved in DMSO/H_2O (6:4, 400 μL, 0.1 M oxalic acid). The resulting mixture was incubated at 60 °C for 10 h and cooled to RT. Purification was performed by preparative HPLC using YMC C18 column (20 x 250 mm) with a gradient of 20 to 95% CH_3CN with 0.1% TFA in 28 min. The pure product fractions were pooled and lyophilized to obtain 7.2 mg of **S40** (26% yield). Analytical HPLC and HRMS were used to confirm the purity and exact mass of the product.

HRMS (MALDI) calcd for $C_{107}H_{169}FN_{23}O_{26}S_3$ $[M+H]^+$: 2307.1750, found: 2307.1749.

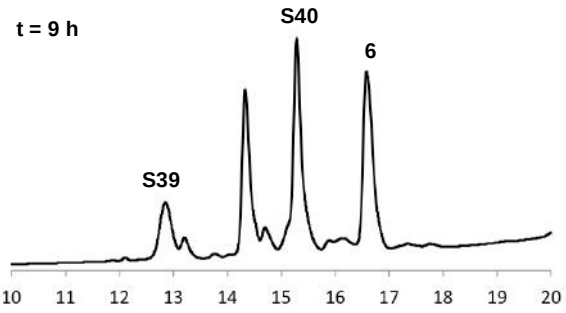


Fig. S81. HPLC monitoring of the KAHA ligation to form **S40**

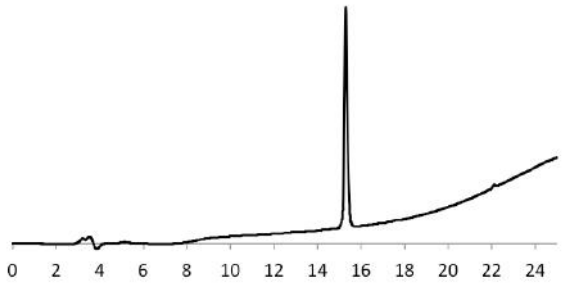


Fig. S82. Analytical HPLC of purified **S40**

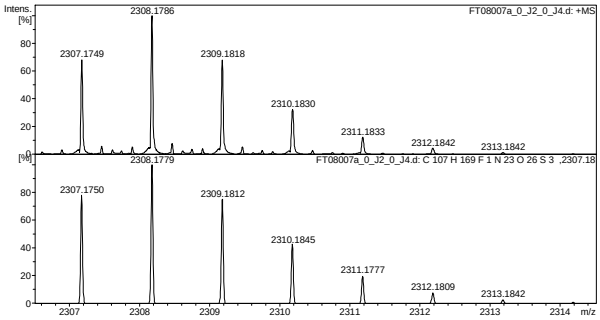
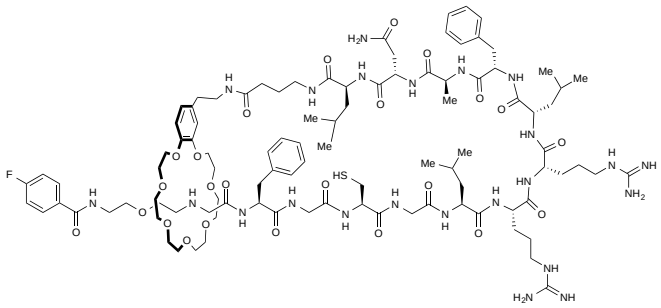


Fig. S83. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **S40**

Lasso peptide S41



S41

4-Mercaptophenylacetic acid (3.5 mg, 21 μmol, 10 equiv) and TCEP-HCl (12 mg, 42 μmol, 20 equiv) were dissolved in 0.25 M sodium phosphate buffer (pH 8.0)/CH_3CN (1:1, 600 μL), and pH of this solution was adjusted to 7.5 by adding 1 M aq NaOH. A solution of **S40** (4.8 mg, 2.1 μmol, 1.0 equiv) in 0.1 M sodium phosphate buffer (pH 7.4)/CH_3CN (1:1, 90 μL) was prepared, and a portion (30 μL) of this solution was added to the ligation buffer. The mixture was incubated at RT for 20 min, and the next portion (30 μL) of the solution of **S40** was added to the mixture. This addition-incubation process was repeated every 20 minutes. After complete addition, the mixture was further incubated at RT for 1 h and purified by preparative HPLC using YMC C18 column (20 x 250 mm) with a gradient of 20 to 95% CH_3CN with 0.1% TFA in 28 min. The pure product fractions were pooled and lyophilized to obtain 1.5 mg of **S41** (34% yield). Analytical HPLC and HRMS were used to confirm the purity and exact mass of the product.

HRMS (MALDI) calcd for $C_{99}H_{153}FN_{23}O_{24}S$ $[M+H]^+$: 2099.1158, found: 2099.1167.

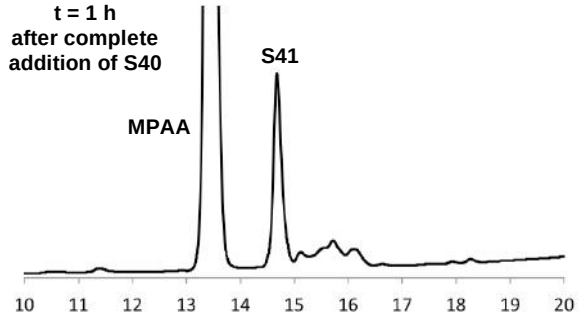


Fig. S84. HPLC monitoring of the NCL to form **S41**

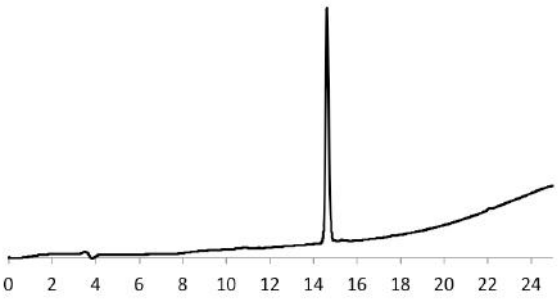


Fig. S85. Analytical HPLC of purified **S41**

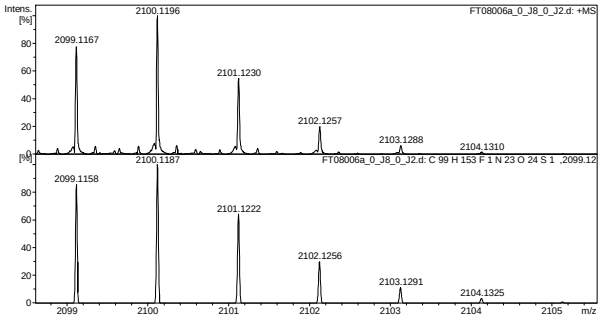
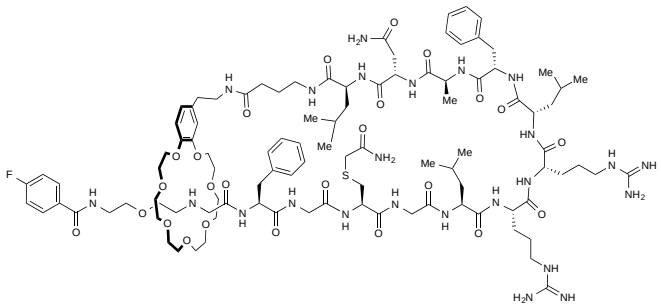


Fig. S86. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **S41**

Cys-alkylated lasso peptide 11



11

To a solution of **S41** (3.1 mg, 1.5 μmol, 1.0 equiv) in 0.1 M sodium phosphate buffer (pH 7.4)/CH_3CN (1:1, 117 μL) was added a solution of iodoacetamide (0.33 mg, 1.8 μmol, 1.2 equiv) in 0.1 M sodium phosphate buffer (pH 7.4)/CH_3CN (1:1, 33 μL). The mixture was incubated at RT for 50 min and purified by preparative HPLC using YMC C18 column (20 x 250 mm) with a gradient of 20 to 95% CH_3CN with 0.1% TFA in 28 min. The pure product fractions were pooled and lyophilized to obtain 1.7 mg of **11** (53% yield). Analytical HPLC and HRMS were used to confirm the purity and exact mass of the product.

HRMS (MALDI) calcd for $C_{101}H_{156}FN_{24}O_{25}S$ $[M+H]^+$: 2156.1373, found: 2156.1304.

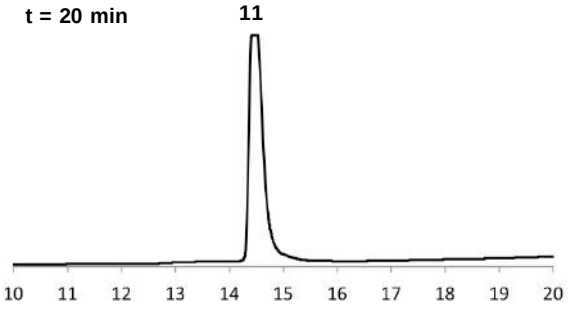


Fig. S87. HPLC monitoring of the cysteine alkylation to form **11**

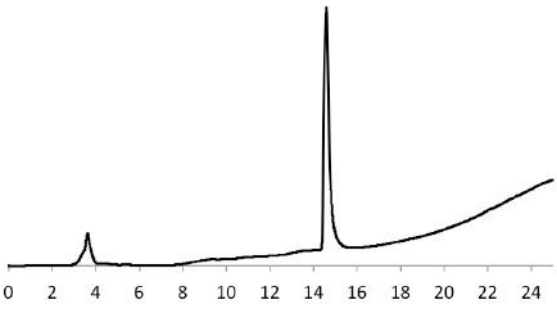


Fig. S88. Analytical HPLC of purified **11**

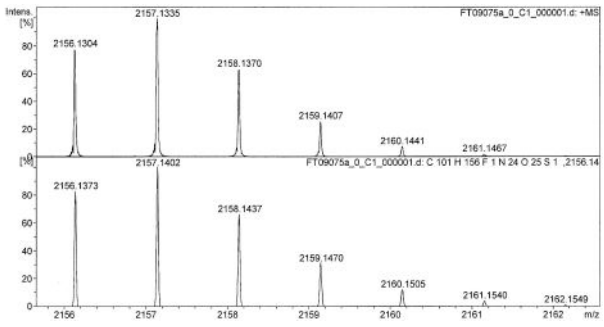


Fig. S89. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **11**

12.2.2. Trypsin digestion of 11

In order to briefly confirm the formation of **11**, trypsin digestion of **11** was conducted. A solution of **11** in H_2O (1 μg/μL) and a solution of trypsin in H_2O (1 μg/μL) were prepared. A solution of 20 μg of peptide was diluted with 50 μL of a buffer (50 mM Tris-HCl, 1 mM $CaCl_2$, pH 7.6). To this solution was added a solution of 1 μg of trypsin. The mixture was incubated at RT for 4 h. The major degradation product was analyzed and identified as [2]rotaxane **S42** by HRMS (MALDI) analysis.

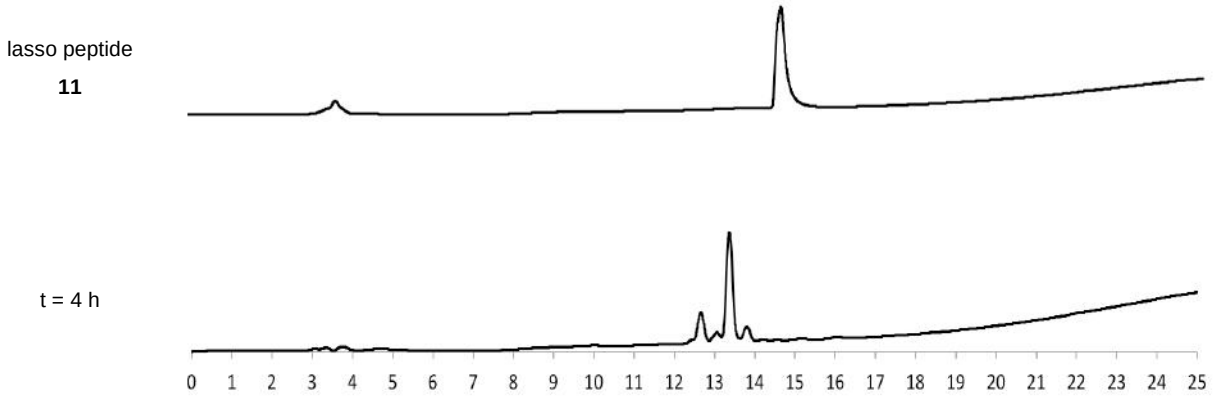
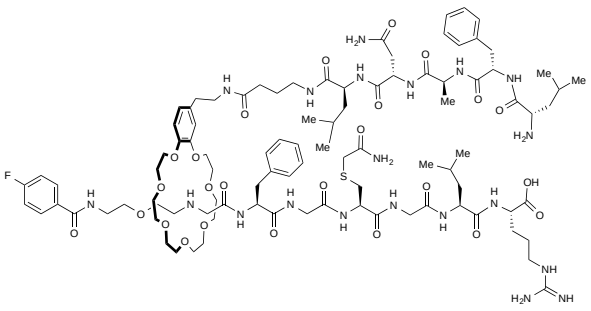


Fig. S90. Trypsin digestion of **11**

Degradation product **S42**



HRMS (MALDI) calcd for $C_{95}H_{146}FN_{20}O_{25}S$ $[M+H]^+$: 2018.0467, found: 2018.0472.

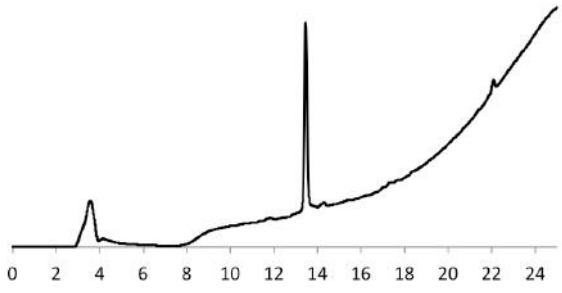


Fig. S91. Analytical HPLC of purified **S42**

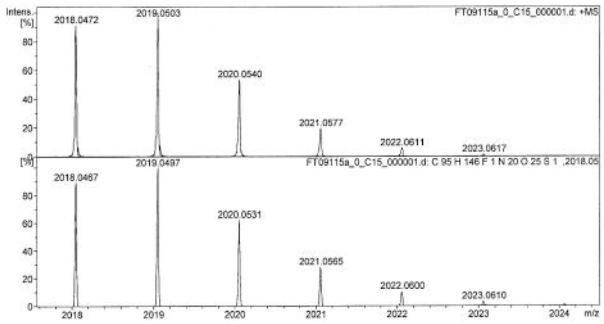
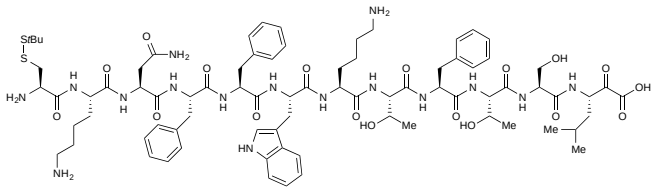


Fig. S92. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **S42**

12.3. Peptide sequence from somatostatin

12.3.1. Synthesis of lasso peptide 12

Peptide α-ketoacid S43



S43

Peptide α-ketoacid **S43** was prepared using the protected leucine α-ketoacid resin **S1** on a 0.10 mmol scale (0.50 g) with a substitution capacity of 0.20 mmol/g. After the full assembly of amino acids, the resin was treated with (95:2.5:2.5) TFA:DODT:H_2O for 1.5 h and removed by filtration. The volatiles were evaporated from the filtrate under reduced pressure. The residue was triturated with Et_2O and centrifuged to obtain the crude **S43**. Purification of crude **S43** was performed by preparative HPLC using YMC C18 column (20 x 250 mm) with a gradient of 20 to 95% CH_3CN with 0.1% TFA in 28 min. The pure product fractions were pooled and lyophilized to obtain 43 mg of **S43** (26% yield for peptide synthesis, resin cleavage and purification steps). Analytical HPLC and HRMS were used to confirm the purity and exact mass of the product.

HRMS (MALDI) calcd for $C_{79}H_{113}N_{16}O_{18}S_2$ $[M+H]^+$: 1637.7855, found: 1637.7863.

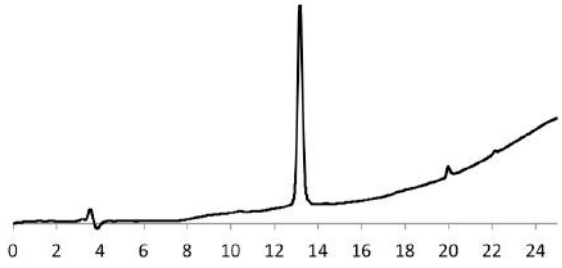


Fig. S93. Analytical HPLC of purified **S43**

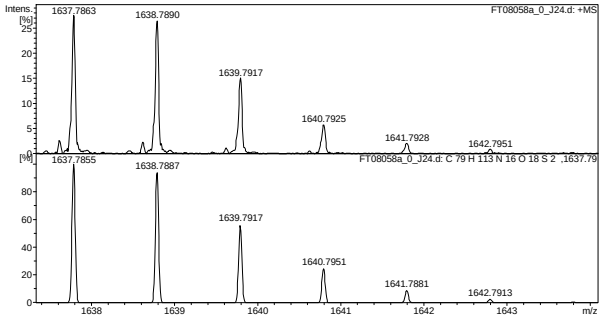
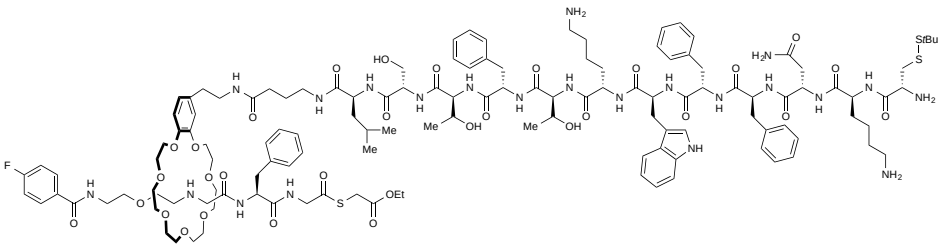


Fig. S94. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **S43**

Peptido[2]rotaxane S44



S44

Hydroxylamine **6** (11 mg, 9.3 μmol, 1.0 equiv) and α-ketoacid **S43** (23 mg, 14 μmol, 1.5 equiv) were dissolved in DMSO/H_2O (6:4, 465 μL, 0.1 M oxalic acid). The resulting mixture was incubated at 60 °C for 23 h and cooled to RT. Purification was performed by preparative HPLC using YMC C18 column (20 x 250 mm) with a gradient of 20 to 95% CH_3CN with 0.1% TFA in 28 min. The pure product fractions were pooled and lyophilized to obtain 7.3 mg of **S44** (29% yield). Analytical HPLC and HRMS were used to confirm the purity and exact mass of the product.

HRMS (MALDI) calcd for $C_{130}H_{186}FN_{22}O_{31}S_3$ $[M+H]^+$: 2666.2795, found: 2666.2788.

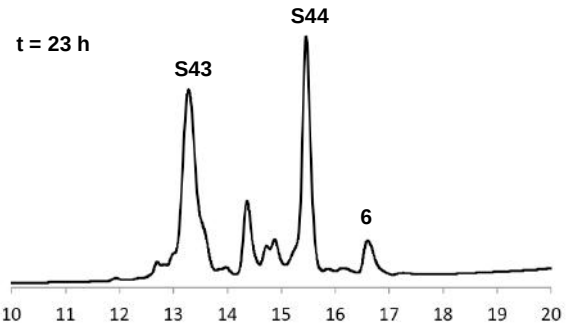


Fig. S95. HPLC monitoring of the KAHA ligation to form **S44**

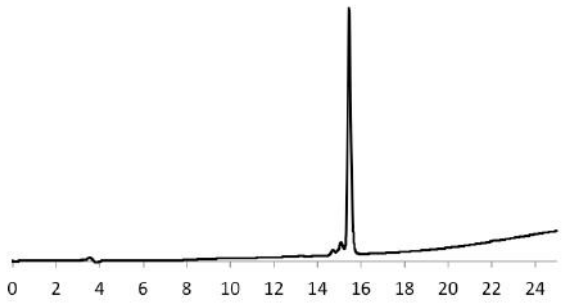


Fig. S96. Analytical HPLC of purified **S44**

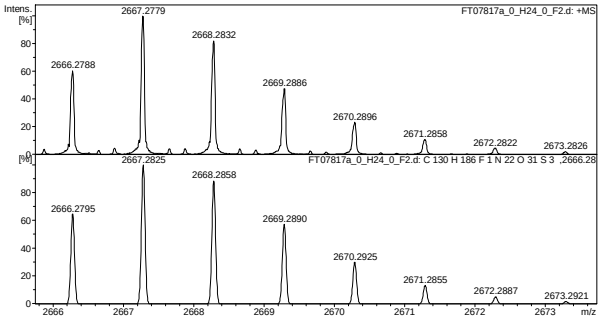
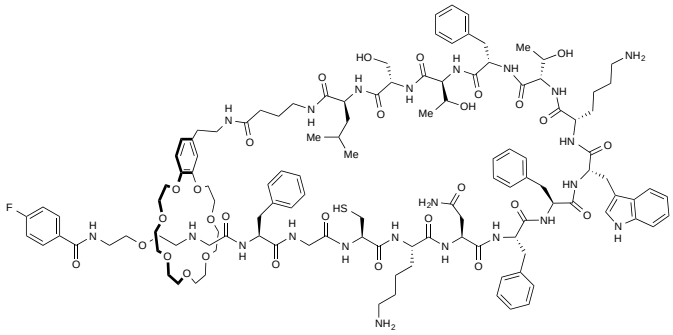


Fig. S97. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **S44**

Lasso peptide S45



S45

4-Mercaptophenylacetic acid (6.6 mg, 38 μmol, 20 equiv) and TCEP-HCl (11 mg, 38 μmol, 20 equiv) were dissolved in 0.25 M sodium phosphate buffer (pH 8.0)/CH_3CN (1:1, 1.0 mL), and pH of this solution was adjusted to 7.8 by adding 1 M aq NaOH. A solution of **S44** (5.2 mg, 1.9 μmol, 1.0 equiv) in 0.1 M sodium phosphate buffer (pH 7.4)/CH_3CN (1:1, 0.20 mL) was prepared, and a portion (50 μL) of this solution was added to the ligation buffer. The mixture was incubated at 40 °C for 10 min, and the next portion (50 μL) of the solution of **S44** was added to the mixture. This addition-incubation process was repeated every 10 minutes. After complete addition, the mixture was further incubated at 40 °C for 1.5 h and purified by preparative HPLC using YMC C18 column (20 x 250 mm) with a gradient of 20 to 95% CH_3CN with 0.1% TFA in 28 min. The pure product fractions were pooled and lyophilized to obtain 2.2 mg of **S45** (47% yield). Analytical HPLC and HRMS were used to confirm the purity and exact mass of the product.

HRMS (MALDI) calcd for $C_{122}H_{169}FN_{22}NaO_{29}S$ $[M+Na]^+$: 2480.2023, found: 2480.2052.

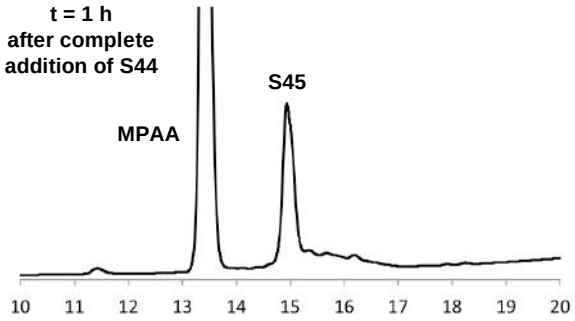


Fig. S98. HPLC monitoring of the NCL to form **S45**

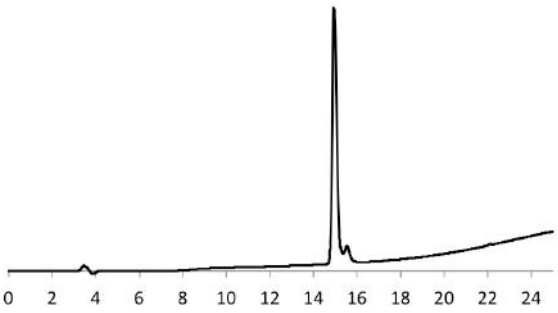


Fig. S99. Analytical HPLC of purified **S45**

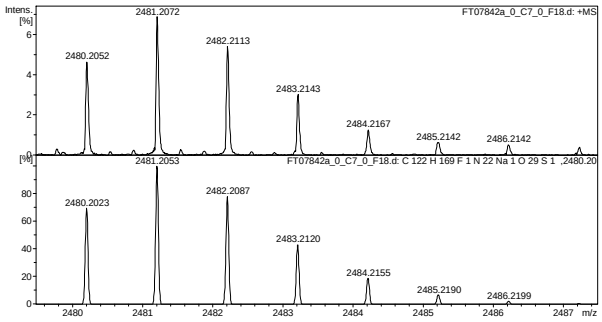
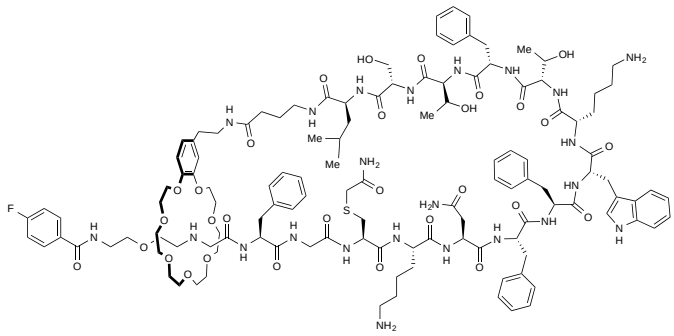


Fig. S100. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **S45**

Cys-alkylated lasso peptide 12



12

To a solution of **S45** (1.4 mg, 0.57 μmol, 1.0 equiv) in 0.1 M sodium phosphate buffer (pH 7.4)/CH_3CN (1:1, 277 μL) was added a solution of iodoacetamide (0.13 mg, 0.68 μmol, 1.2 equiv) in 0.1 M sodium phosphate buffer (pH 7.4)/CH_3CN (1:1, 13 μL). The mixture was incubated at RT for 20 min and purified by preparative HPLC using YMC C18 column (20 x 250 mm) with a gradient of 20 to 95% CH_3CN with 0.1% TFA in 28 min. The pure product fractions were pooled and lyophilized to obtain 0.9 mg of **12** (63% yield). Analytical HPLC and HRMS were used to confirm the purity and exact mass of the product.

HRMS (MALDI) calcd for $C_{124}H_{173}FN_{23}O_{30}S$ $[M+H]^+$: 2515.2418, found: 2515.2424.

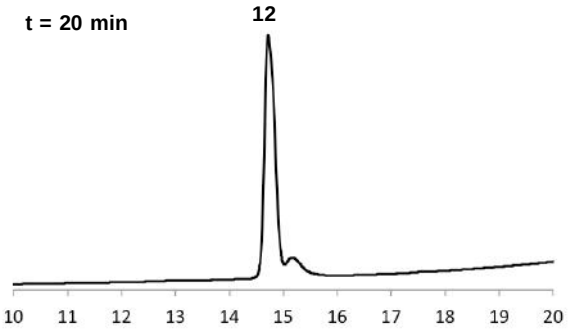


Fig. S101. HPLC monitoring of the cysteine alkylation to form **12**

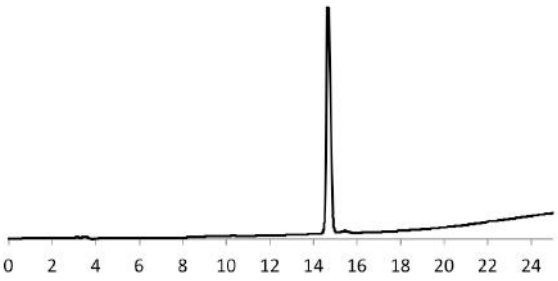


Fig. S102. Analytical HPLC of purified **12**

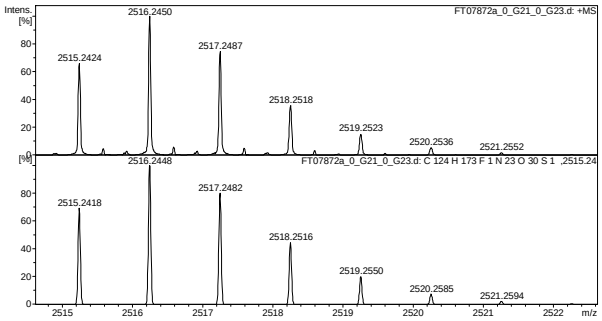


Fig. S103. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **12**

12.3.2. Trypsin digestion of 12

In order to briefly confirm the formation of **12**, trypsin digestion of **12** was conducted. A solution of **12** in H_2O (1 μg/μL) and a solution of trypsin in H_2O (1 μg/μL) were prepared. A solution of 20 μg of peptide was diluted with 50 μL of a buffer (50 mM Tris-HCl, 1 mM $CaCl_2$, pH 7.6). To this solution was added a solution of 1 μg of trypsin. The mixture was incubated at RT for 4 h. The starting peptide was degraded to two products, and these were identified as linear peptide **S46** and [2]rotaxane **S47** by HRMS (MALDI) analysis. This result strongly supported the formation of **12**.

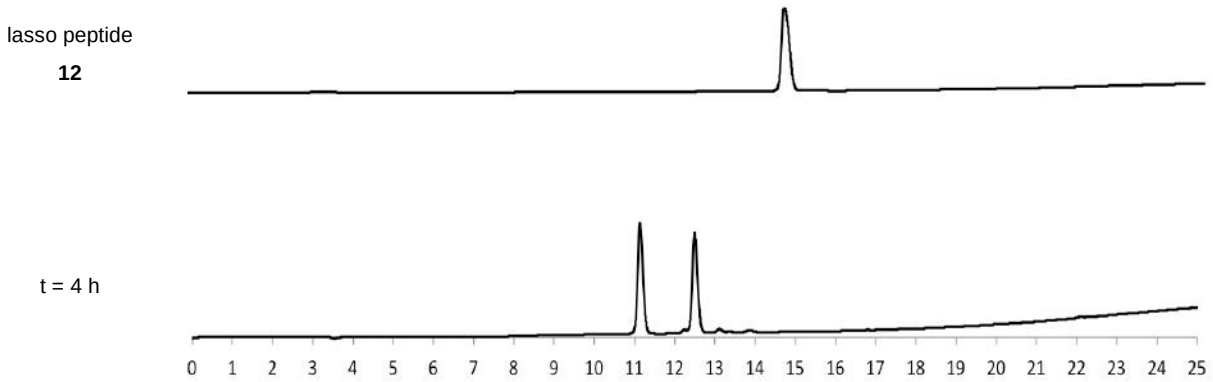
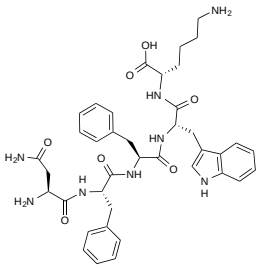


Fig. S104. Trypsin digestion of **12**

Degradation product **S46**



HRMS (MALDI) calcd for $C_{39}H_{49}N_8O_7$ $[M+H]^+$: 741.3719, found: 741.3718.

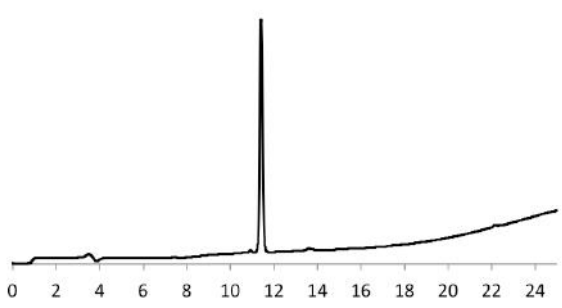


Fig. S105. Analytical HPLC of purified **S46**

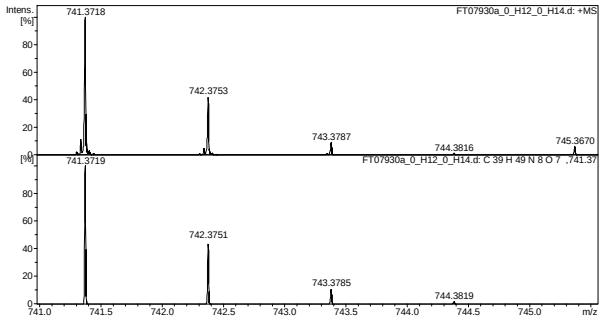
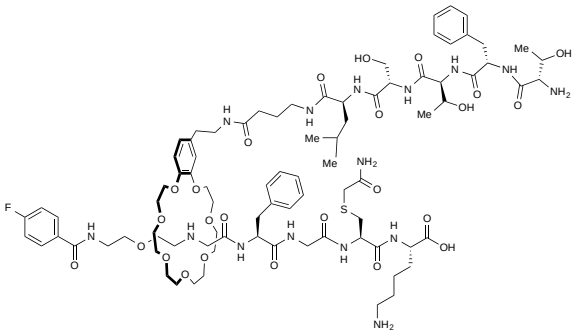


Fig. S106. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **S46**

Degradation product **S47**



HRMS (MALDI) calcd for $C_{85}H_{129}FN_{15}O_{25}S$ $[M+H]^+$: 1810.8983, found: 1810.8970.

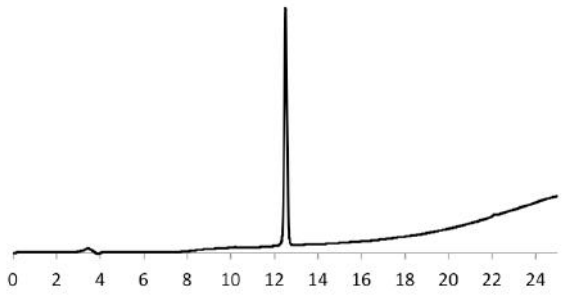


Fig. S107. Analytical HPLC of purified **S47**

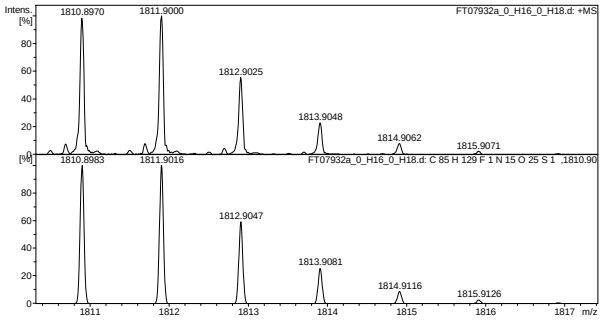
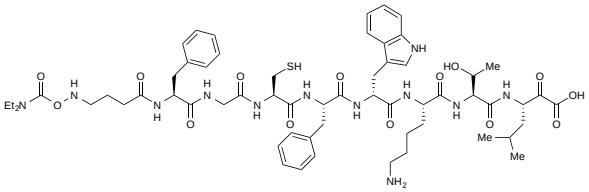


Fig. S108. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **S47**

13. Synthesis of cyclic peptide C1

13.1. Bifunctional linear peptide S48



S48

Bifunctional linear peptide **S48** was prepared using the protected leucine α-ketoacid resin **S1** on a 0.22 mmol scale (1.2 g) with a substitution capacity of 0.18 mmol/g. In addition to Fmoc amino acids listed in 1.4., *N*-Boc hydroxylamine **S13** was used for this peptide synthesis. The resin was treated with (95:2.5:2.5) TFA:DODT:H_2O for 1 h and removed by filtration. The volatiles were evaporated from the filtrate under reduced pressure. The residue was triturated with Et_2O and centrifuged to obtain the crude **S48**. Purification of crude **S48** was performed by preparative HPLC using YMC C18 column (20 x 250 mm) with a gradient of 20 to 95% CH_3CN with 0.1% TFA in 28 min. The pure product fractions were pooled and lyophilized to obtain 20.9 mg of **S48** (8% yield for peptide synthesis, resin cleavage and purification steps). Analytical HPLC and HRMS were used to confirm the purity and exact mass of the product.

HRMS (MALDI) calcd for $C_{60}H_{85}N_{12}O_{14}S$ $[M+H]^+$: 1229.6023, found: 1229.6019.

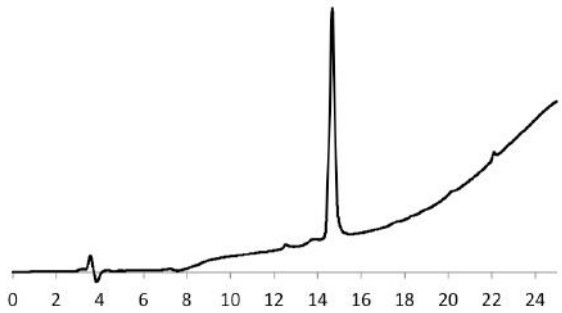


Fig. S109. Analytical HPLC of purified **S48**

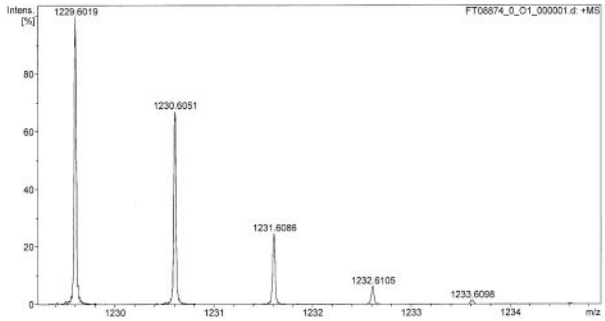
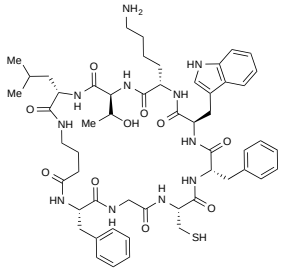


Fig. S110. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **S48**

13.2. Cyclic peptide S49



S49

Bifunctional linear peptide **S48** (12 mg, 9.9 μmol) was dissolved in CH_3CN/H_2O (2:1, 3.3 mL, 0.1 M oxalic acid). The mixture was incubated at 70 °C for 7 h and cooled to RT. Purification was

performed by preparative HPLC using YMC C18 column (20 x 250 mm) with a gradient of 20 to 95% CH_3CN with 0.1% TFA in 28 min. The pure product fractions were pooled and lyophilized to obtain 4.4 mg of **S49** (42% yield). Analytical HPLC and HRMS were used to confirm the purity and exact mass of the product.

HRMS (MALDI) calcd for $C_{54}H_{74}N_{11}O_{10}S$ $[M+H]^+$: 1068.5335, found: 1068.5332.

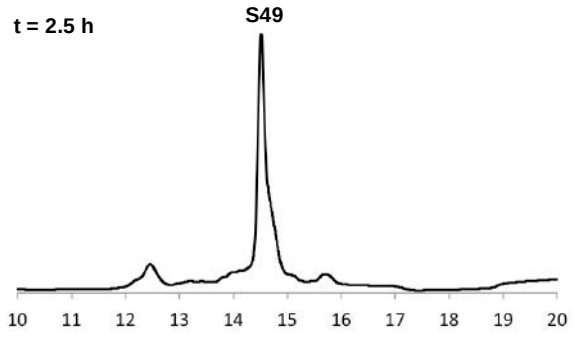


Fig. S111. HPLC monitoring of the KAHA cyclization to form **S49**

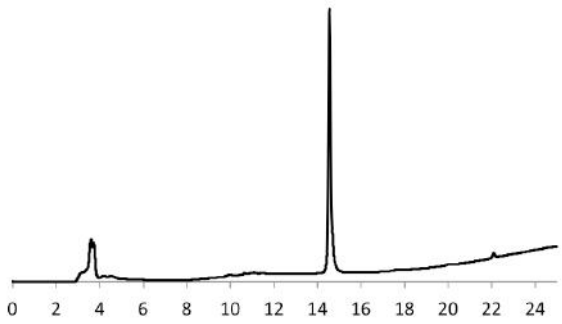


Fig. S112. Analytical HPLC of purified **S49**

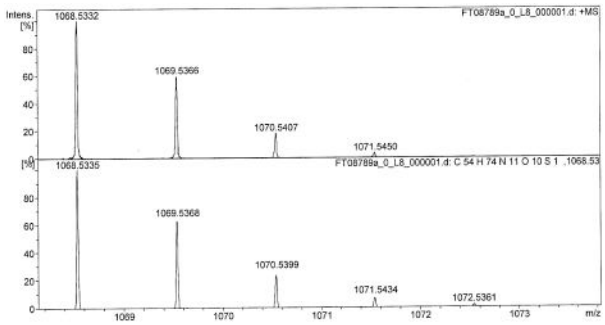
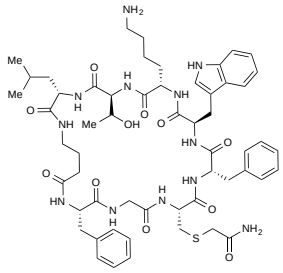


Fig. S113. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **S49**

13.3. Cyclic peptide C1



C1

To a solution of **S49** (4.4 mg, 4.1 μmol, 1.0 equiv) in 0.1 M sodium phosphate buffer (pH 7.4)/CH_3CN (1:1, 319 μL) was added a solution of iodoacetamide (0.91 mg, 4.9 μmol, 1.2 equiv) in CH_3CN (91 μL). The mixture was incubated at 37 °C for 2 h. Purification was performed by preparative HPLC using YMC C18 column (20 x 250 mm) with a gradient of 20 to 95% CH_3CN with 0.1% TFA in 28 min. The pure product fractions were pooled and lyophilized to obtain 1.5 mg of **C1** (33% yield). Analytical HPLC and HRMS were used to confirm the purity and exact mass of the product.

HRMS (MALDI) calcd for $C_{56}H_{77}N_{12}O_{11}S$ $[M+H]^+$: 1125.5550, found: 1125.5547.

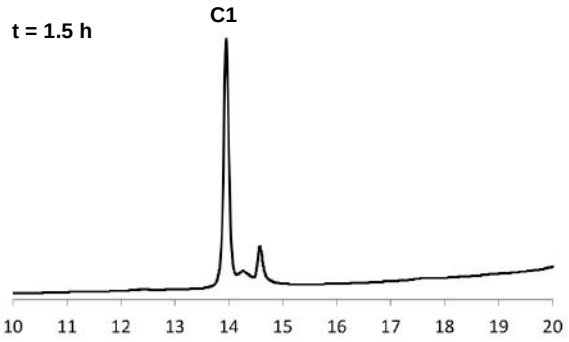


Fig. S114. HPLC monitoring of the cysteine alkylation to form **C1**

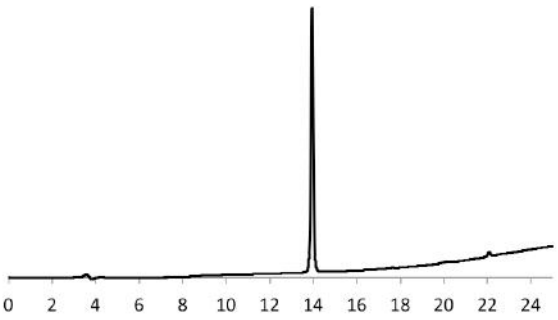


Fig. S115. Analytical HPLC of purified **C1**

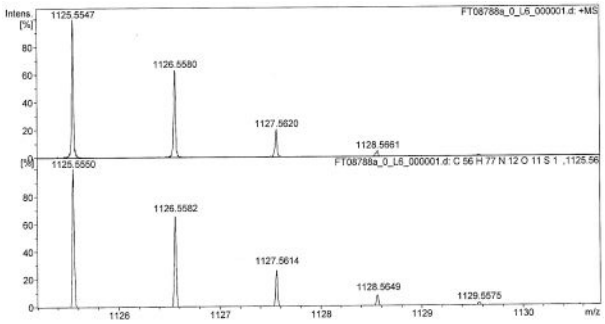


Fig. S116. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **C1**

Investigation of the properties of the lasso peptides

Note: Lyophilized peptides were dissolved in DMSO to make 10 mM stock solutions. The following stability assays were conducted by taking an aliquot from these stock solutions.

14. Thermal stability assay

A solution of 0.1 mg of lasso peptide was diluted with H_2O (10% (v/v) final concentration of DMSO) and incubated at 95 °C for 8 h. Samples were cooled to RT and analyzed by analytical RP-HPLC.

14.1. Lasso peptide L1

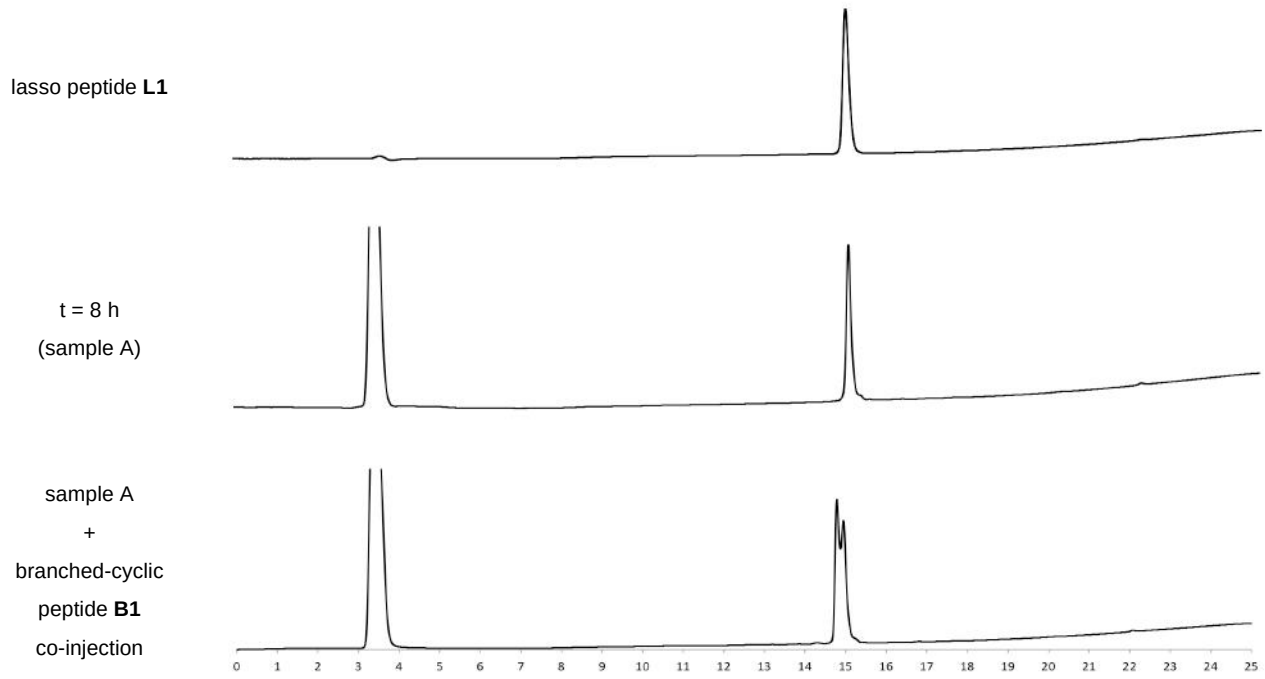


Fig. S117. Thermal stability assay of **L1**

14.2. Lasso peptide L2

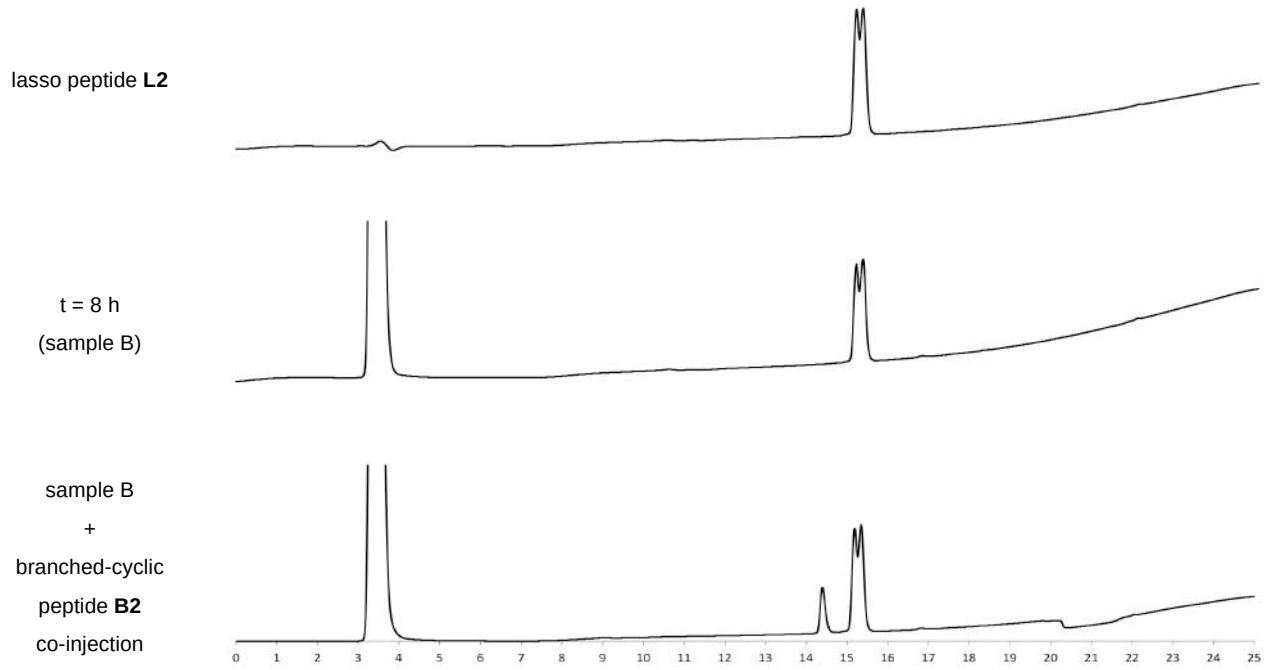


Fig. S118. Thermal stability assay of **L2**

14.3. Lasso peptide L3

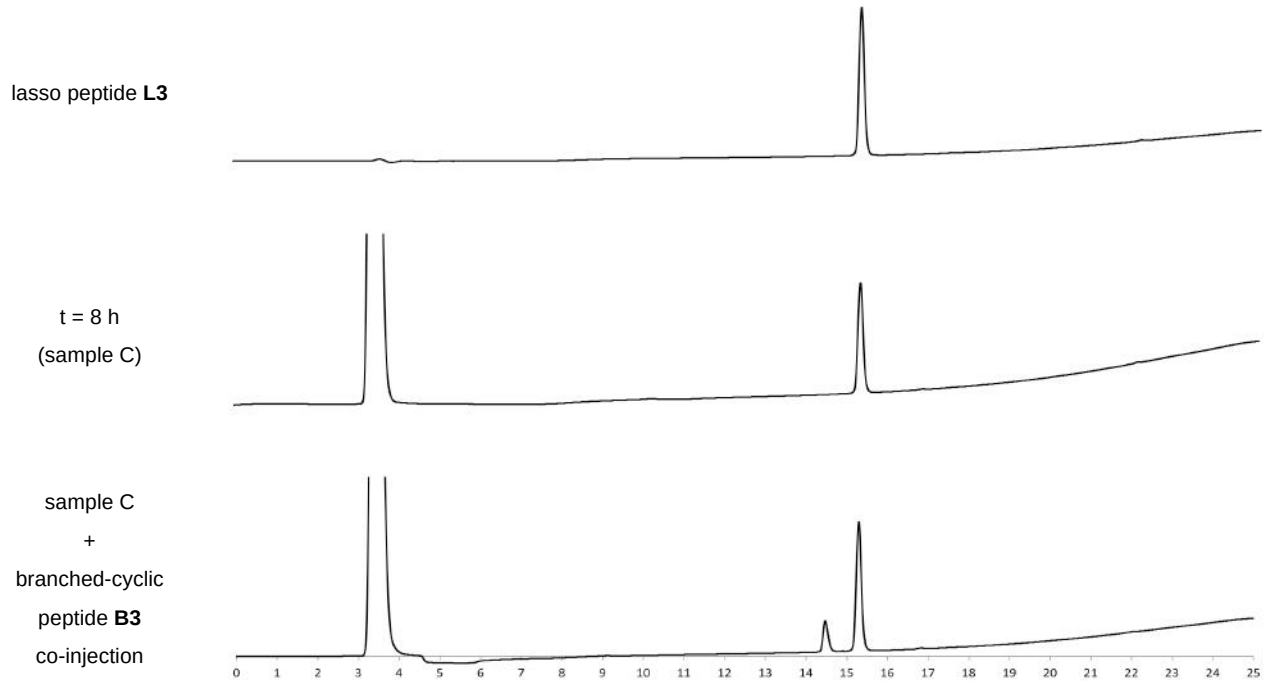


Fig. S119. Thermal stability assay of **L3**

15. Chymotrypsin assay

A solution of 20 μg of peptide was diluted with 51 μL of a buffer (100 mM Tris-HCl, 10 mM $CaCl_2$, pH 8.2). An aliquot (10 μL) was taken from this solution, diluted with H_2O/ CH_3CN (1:1, 10 μL, 0.1% TFA) and injected to analytical RP-HPLC. The peak area of the peptide (A_0) was determined by integration.

A solution of 20 μg of peptide was diluted with 50 μL of a buffer (100 mM Tris-HCl, 10 mM $CaCl_2$, pH 8.2). To this solution was added chymotrypsin dissolved in the same buffer (1.0 μg/μL, 1.0 μL). The mixture was incubated at RT for 24 h and analyzed at selected time points: 2, 4, and 24 h for lasso peptides; 2 and 4 h for branched-cyclic peptides; 2, 4, and 12 h for cyclic peptide. For analysis, an aliquot (10 μL) was taken from the reaction mixture, diluted with H_2O/CH_3CN (1:1, 10 μL, 0.1% TFA) and injected to analytical RP-HPLC. The peak area of the starting peptide (A_t) was determined by integration. Percentage of the remaining peptide at each time point was calculated as follows:

$$Peptide\ remaining\ (\%) = \left({}^{A_t}/_{A_0}\right) \times 100$$

15.1. Lasso peptide L1

15.1.1. Analytical HPLC traces

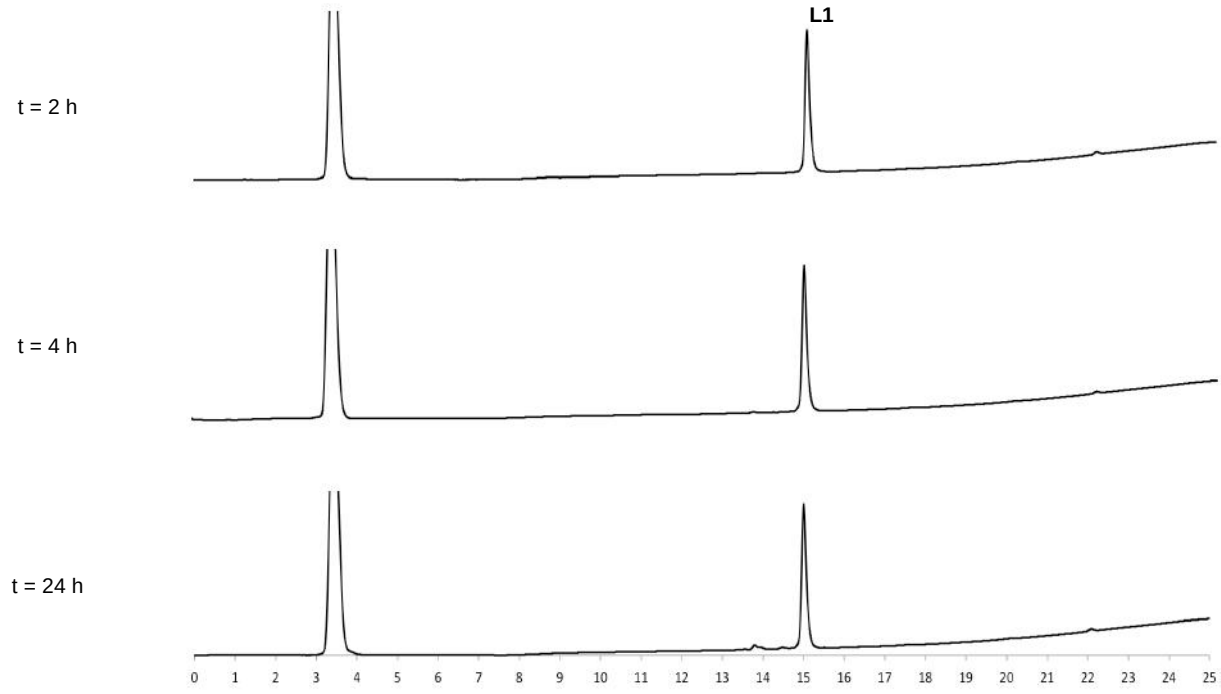


Fig. S120. Chymotrypsin assay of **L1**

15.2. Branched-cyclic peptide B1

15.2.1. Analytical HPLC traces

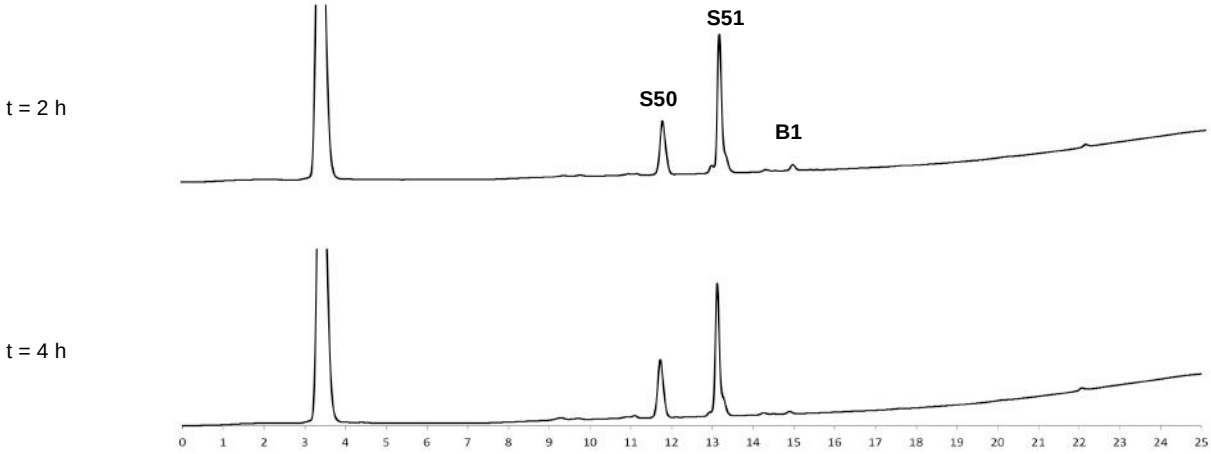
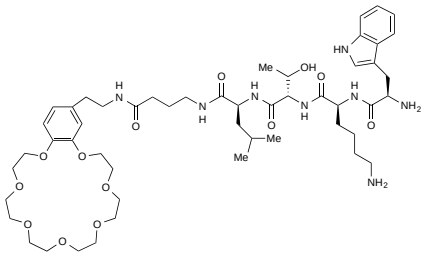


Fig. S121. Chymotrypsin assay of **B1**

15.2.2. Characterization of degradation products

Degradation product **S50**



HRMS (MALDI) calcd for $C_{51}H_{81}N_8O_{13}$ $[M+H]^+$: 1013.5918, found: 1013.5905.

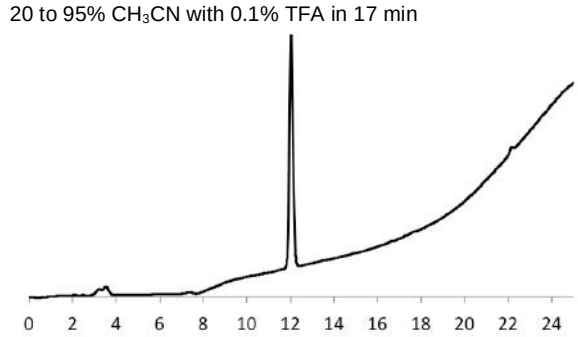


Fig. S122. Analytical HPLC of purified **S50**

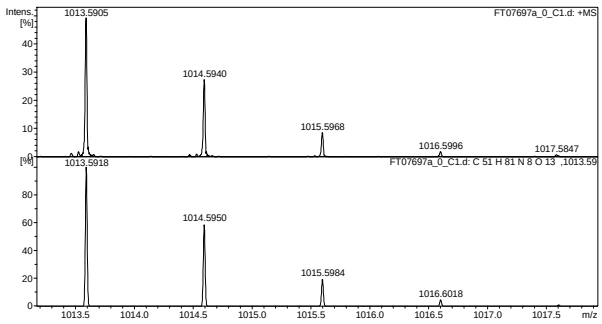
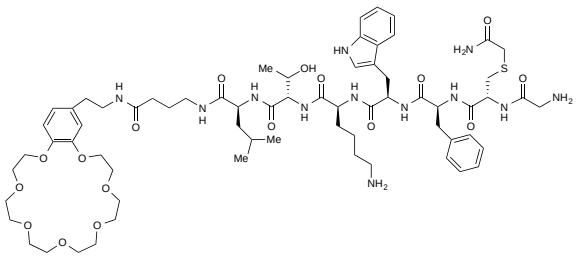


Fig. S123. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **S50**

Degradation product **S51**



HRMS (MALDI) calcd for $C_{67}H_{101}N_{12}O_{17}S$ $[M+H]^+$: 1377.7123, found: 1377.7121.

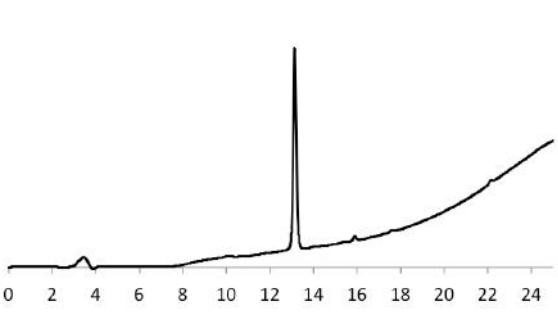


Fig. S124. Analytical HPLC of purified **S51**

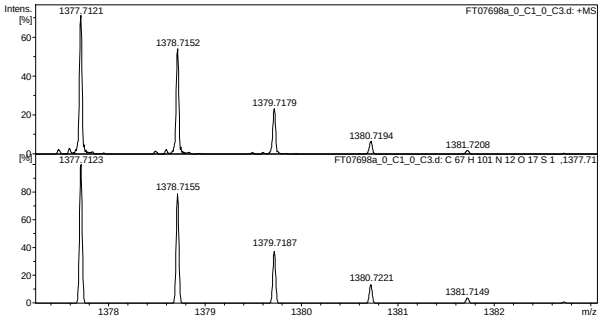


Fig. S125. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **S51**

15.3. Lasso peptide L2

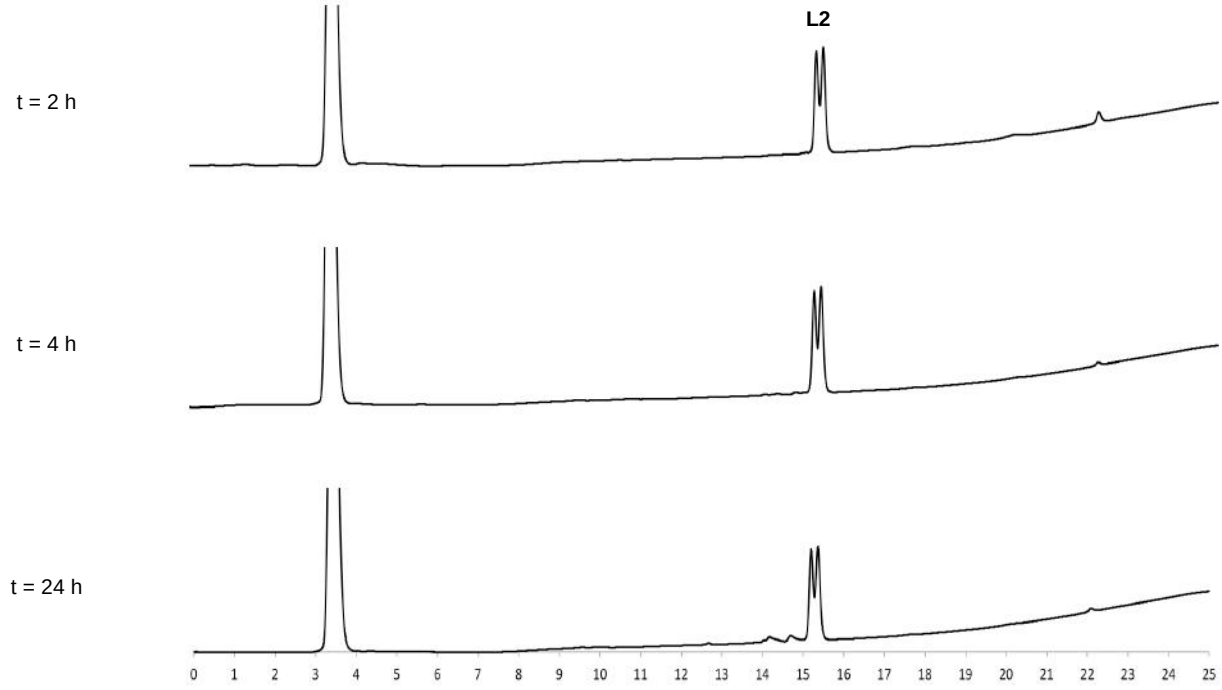


Fig. S126. Chymotrypsin assay of **L2**

15.4. Branched-cyclic peptide B2

15.4.1. Analytical HPLC traces

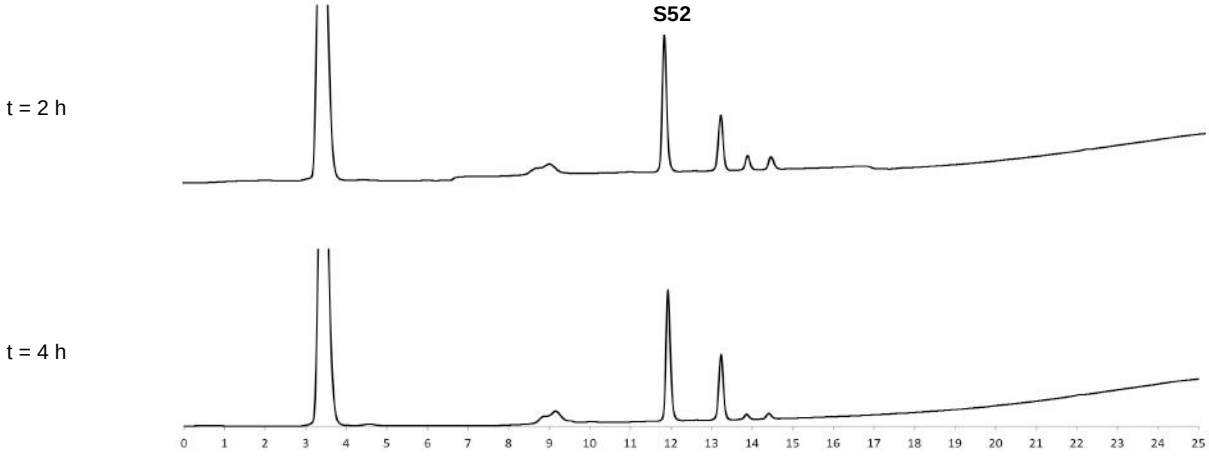
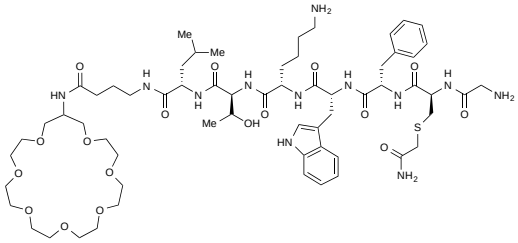


Fig. S127. Chymotrypsin assay of **B2**

15.4.2. Characterization of degradation products

Degradation product **S52**



HRMS (MALDI) calcd for $C_{62}H_{99}N_{12}O_{17}S$ $[M+H]^+$: 1315.6966, found: 1315.6964.

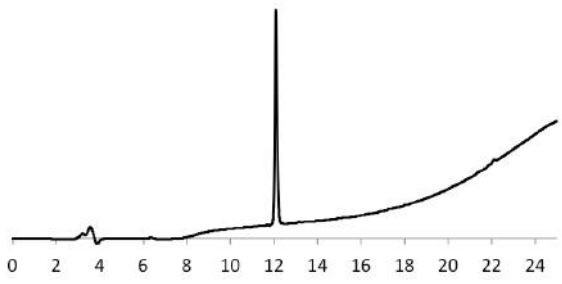


Fig. S128. Analytical HPLC of purified **S52**

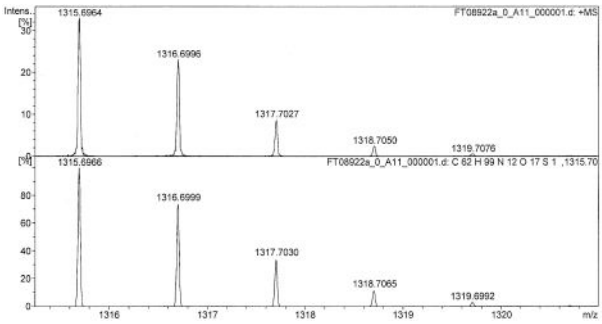


Fig. S129. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **S52**

15.5. Lasso peptide L3

15.5.1. Analytical HPLC traces

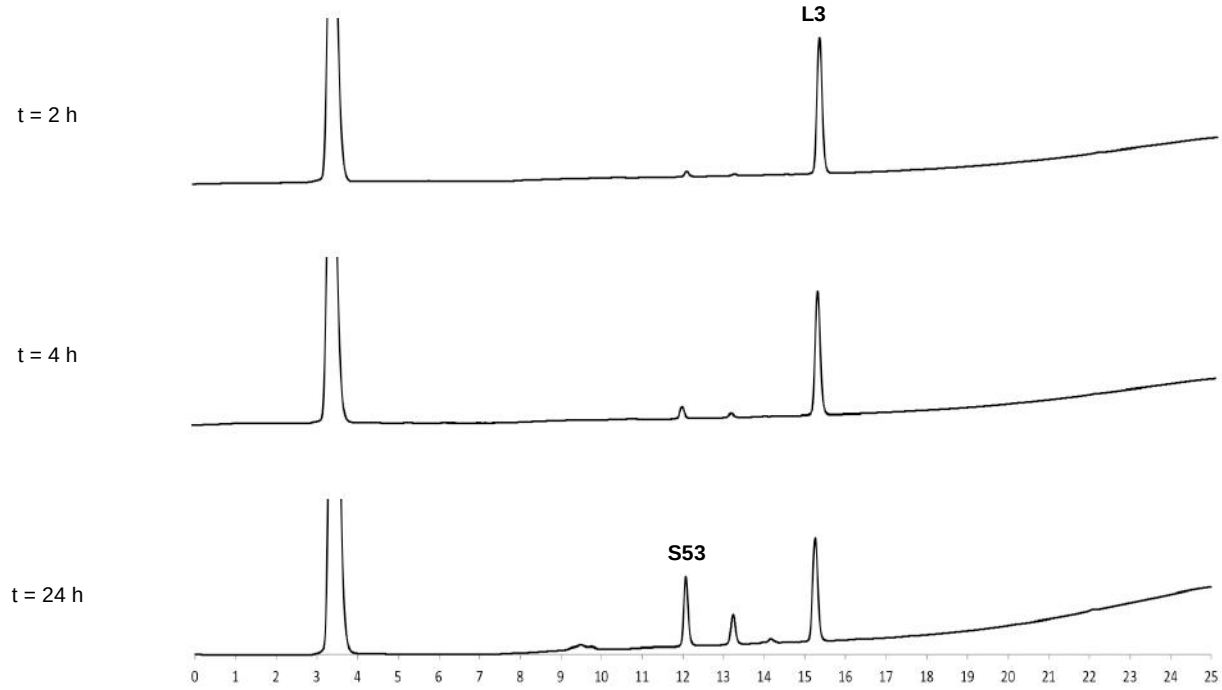
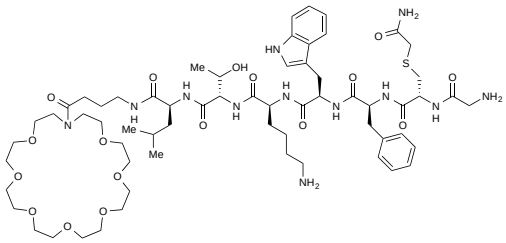


Fig. S130. Chymotrypsin assay of **L3**

15.5.2. Characterization of degradation products

Degradation product **S53**



HRMS (MALDI) calcd for $C_{63}H_{101}N_{12}O_{17}S$ $[M+H]^+$: 1329.7123, found: 1329.7118.

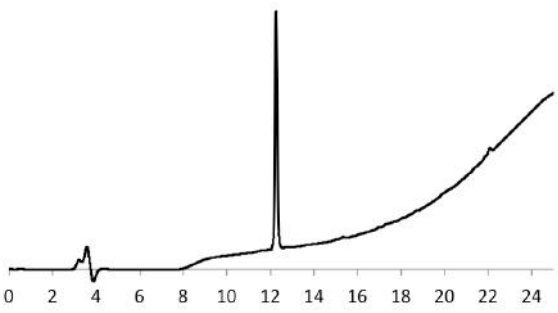


Fig. S131. Analytical HPLC of purified **S53**

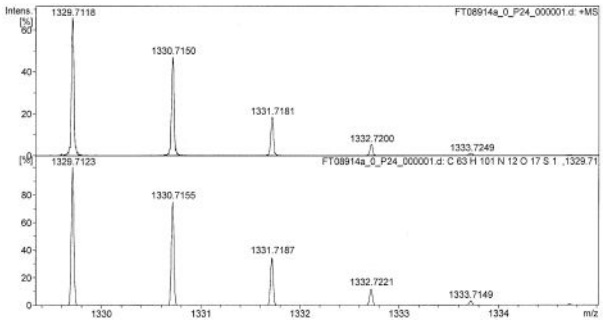


Fig. S132. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **S53**

15.6. Branched-cyclic peptide B3

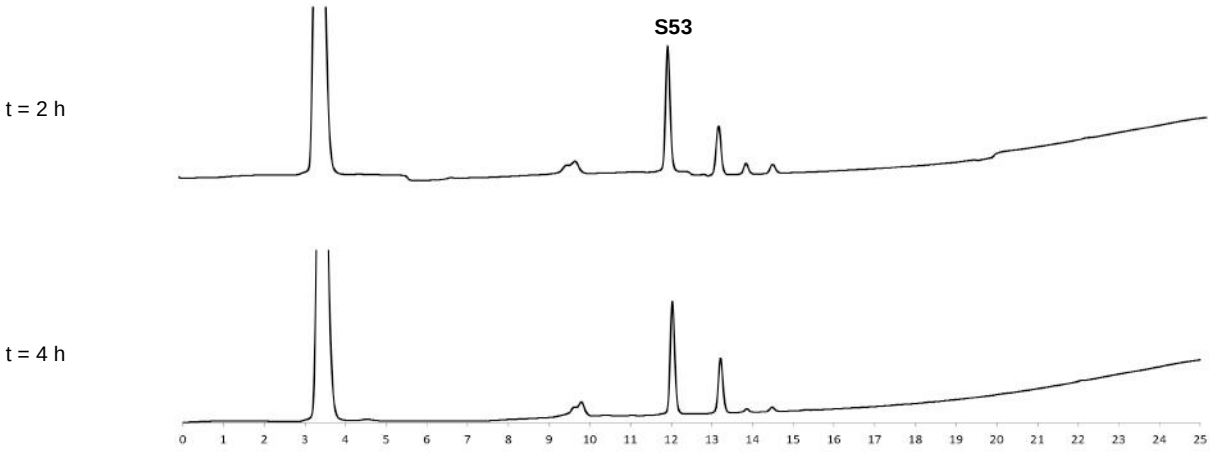


Fig. S133. Chymotrypsin assay of **B3**

15.7. Cyclic peptide C1

15.7.1. Analytical HPLC traces

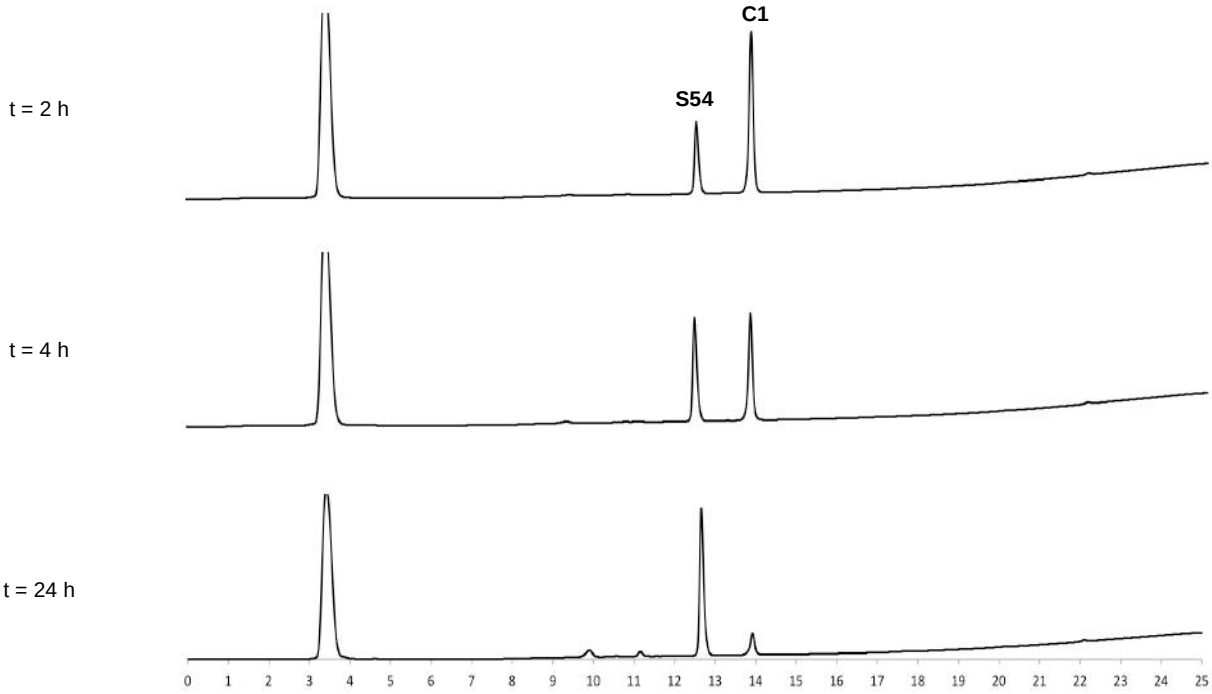
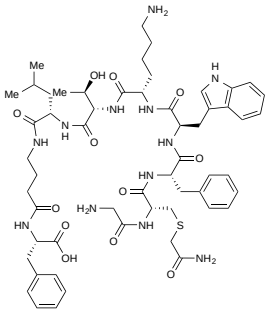


Fig. S134. Chymotrypsin assay of **C1**

15.7.2. Characterization of degradation products

Degradation product **S54** (Shown here is a possible structure based on specificity of chymotrypsin)



HRMS (MALDI) calcd for $C_{56}H_{79}N_{12}O_{12}S$ $[M+H]^+$: 1143.5656, found: 1143.5656.

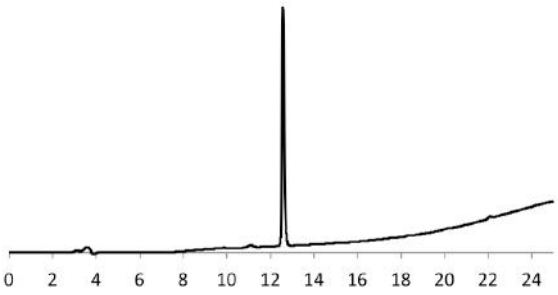


Fig. S135. Analytical HPLC of purified **S54**

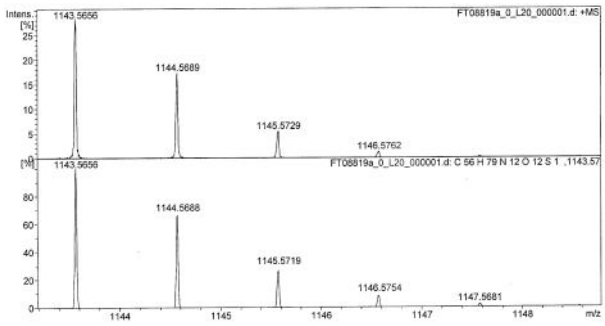


Fig. S136. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **S54**

15.8. Plots of time vs conversion

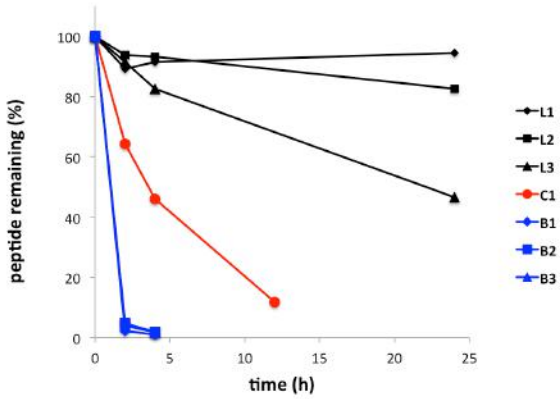


Fig. S137. Plots of time vs conversion (chymotrypsin assay)

16. Trypsin assay

A solution of 20 μg of peptide was diluted with 51 μL of a buffer (50 mM Tris-HCl, 1 mM $CaCl_2$, pH 7.6). An aliquot (10 μL) was taken from this solution, diluted with H_2O/ CH_3CN (1:1, 10 μL, 0.1% TFA) and injected to analytical RP-HPLC. The peak area of the peptide was determined by integration.

A solution of 20 μg of peptide was diluted with 50 μL of a buffer (50 mM Tris-HCl, 1 mM $CaCl_2$, pH 7.6). To this solution was added trypsin dissolved in the same buffer (1.0 μg/μL, 1.0 μL). The mixture was incubated at RT for 8 h and analyzed at selected time points: 2, 4, and 8 h for lasso peptides and cyclic peptide; 2 and 4 h for branched-cyclic peptides. For analysis, an aliquot (10 μL) was taken from the reaction mixture, diluted with H_2O/CH_3CN (1:1, 10 μL, 0.1% TFA) and injected to analytical RP-HPLC. The peak area of the starting peptide was determined by integration. Percentage of the remaining peptide was calculated as described in ESI section 15.

16.1. Lasso peptide L1

16.1.1. Analytical HPLC traces

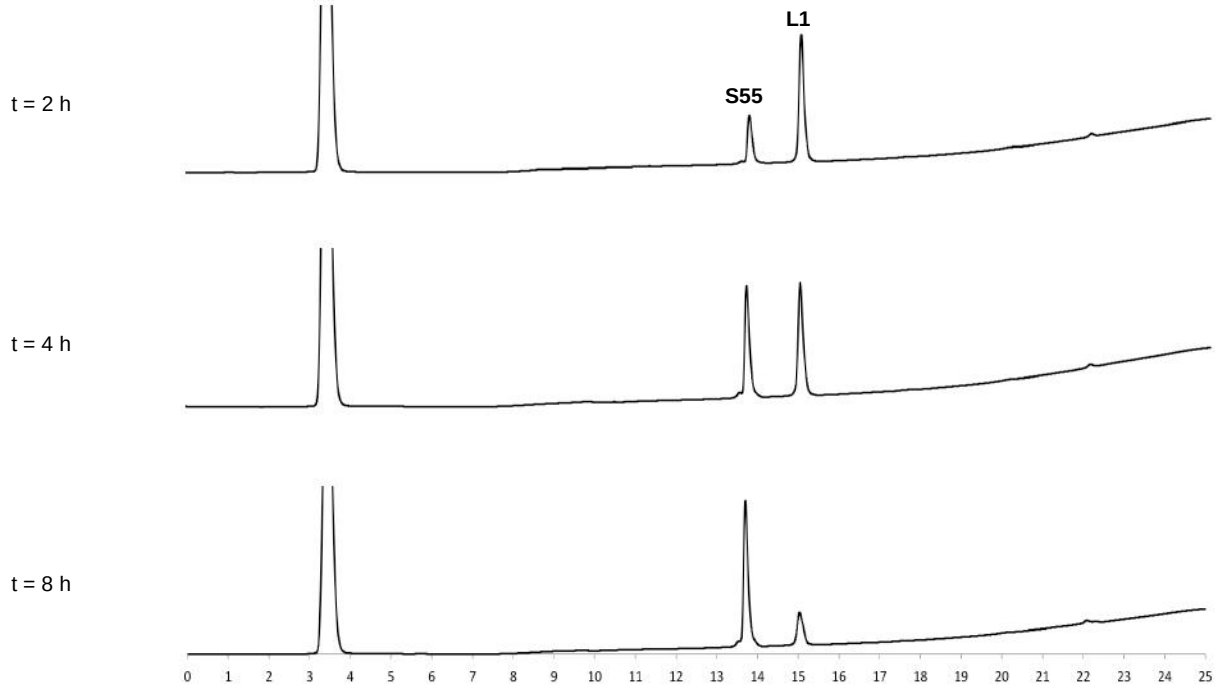
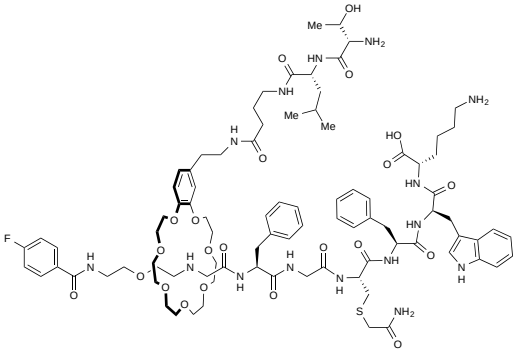


Fig. S138. Trypsin assay of **L1**

16.1.2. Characterization of degradation products

Degradation product **S55** (Shown here is a possible structure based on specificity of trypsin)



HRMS (MALDI) calcd for $C_{89}H_{127}FN_{15}O_{22}S$ $[M+H]^+$: 1808.8979, found: 1808.8985.

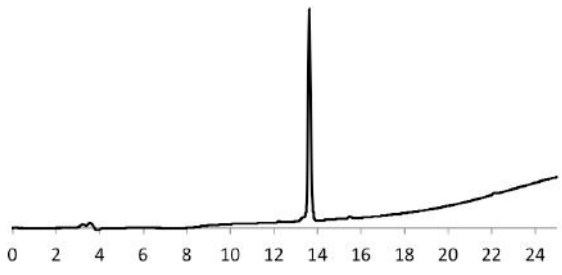


Fig. S139. Analytical HPLC of purified **S55**

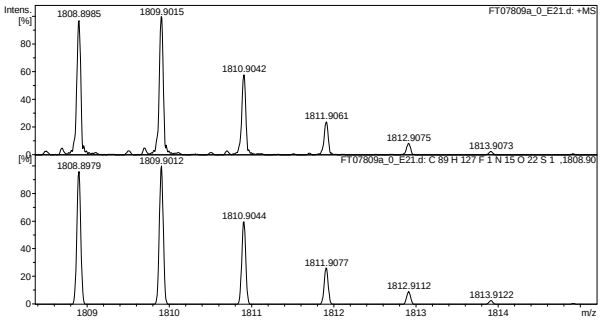


Fig. S140. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **S55**

16.2. Branched-cyclic peptide B1

16.2.1. Analytical HPLC traces

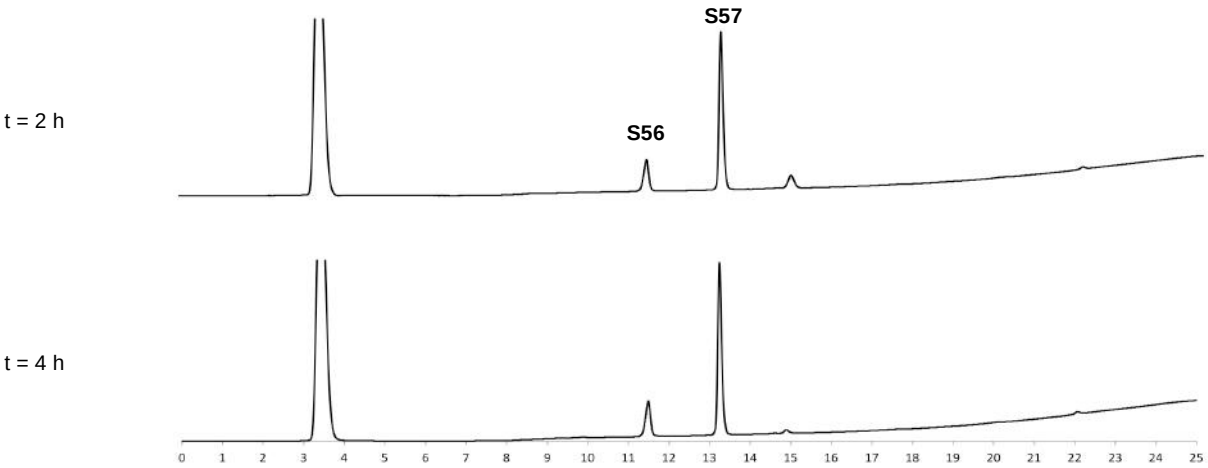
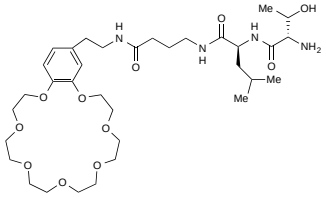


Fig. S141. Trypsin assay of **B1**

16.2.2. Characterization of degradation products

Degradation product **S56**



HRMS (MALDI) calcd for $C_{34}H_{59}N_4O_{11}$ $[M+H]^+$: 699.4175, found: 699.4166.

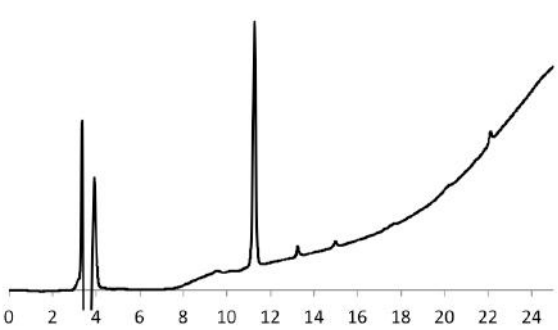


Fig. S142. Analytical HPLC of purified **S56**

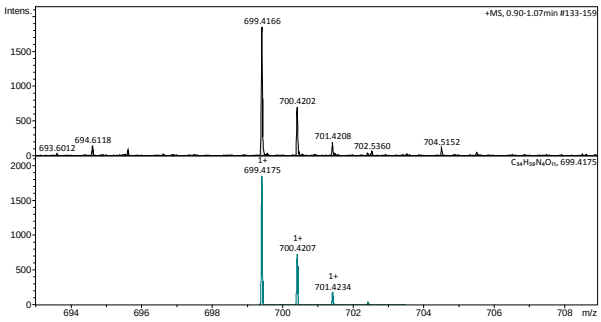
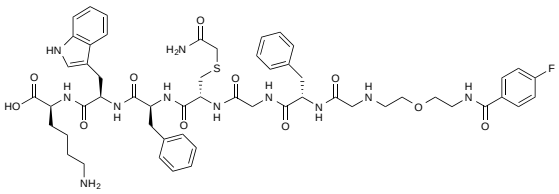


Fig. S143. HRMS (ESI) of measured (top) and calculated (bottom) isotopic pattern of **S56**

Degradation product **S57**



HRMS (MALDI) calcd for $C_{55}H_{69}FN_{11}O_{11}S$ $[M+H]^+$: 1110.4877, found: 1110.4855.

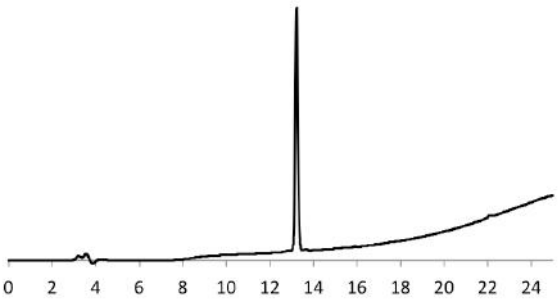


Fig. S144. Analytical HPLC of purified **S57**

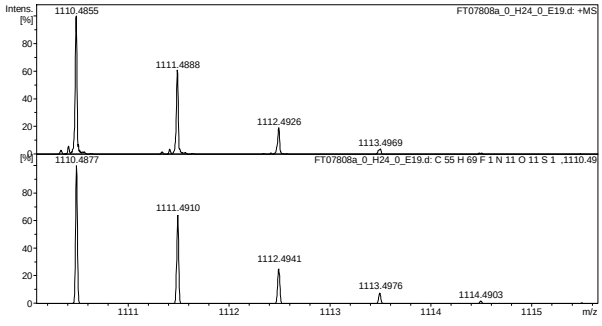


Fig. S145. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **S57**

16.3. Lasso peptide L2

16.3.1. Analytical HPLC traces

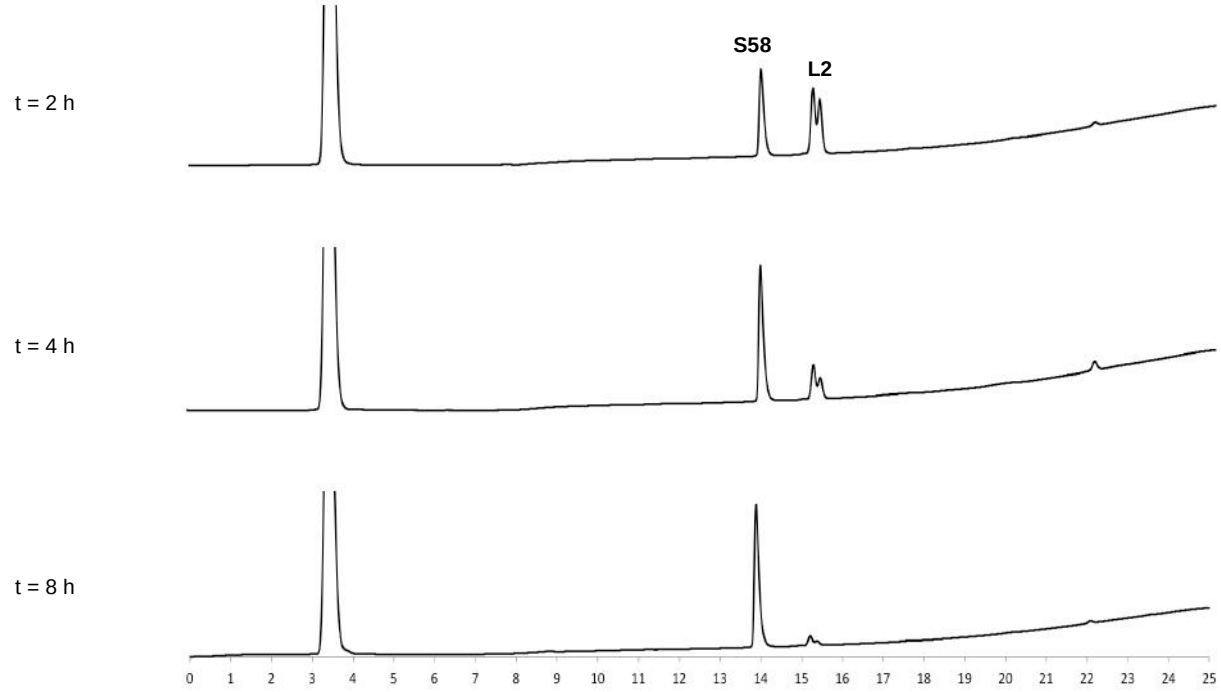
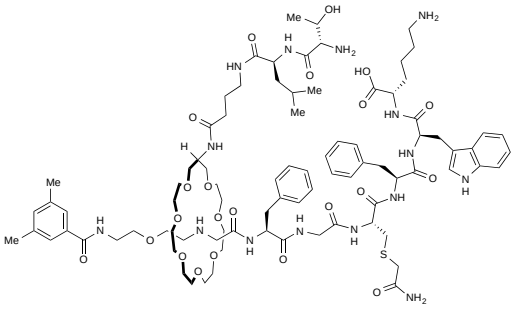


Fig. S146. Trypsin assay of **L2**

16.3.2. Characterization of degradation products

Degradation product **S58** (Shown here is a possible structure based on specificity of trypsin)



HRMS (MALDI) calcd for $C_{86}H_{130}N_{15}O_{22}S$ $[M+H]^+$: 1756.9230, found: 1756.9229.

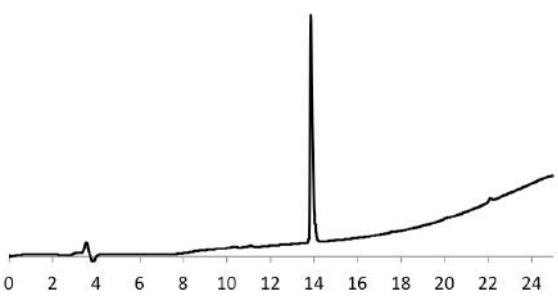


Fig. S147. Analytical HPLC of purified **S58**

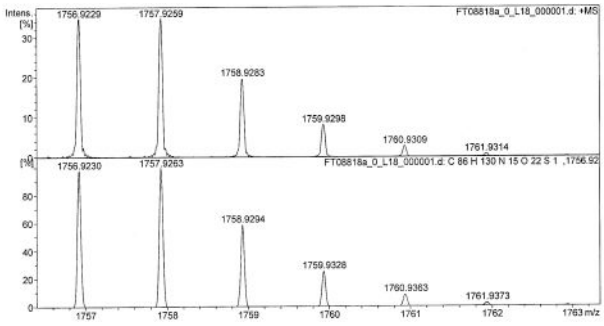


Fig. S148. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **S58**

16.4. Branched-cyclic peptide B2

16.4.1. Analytical HPLC traces

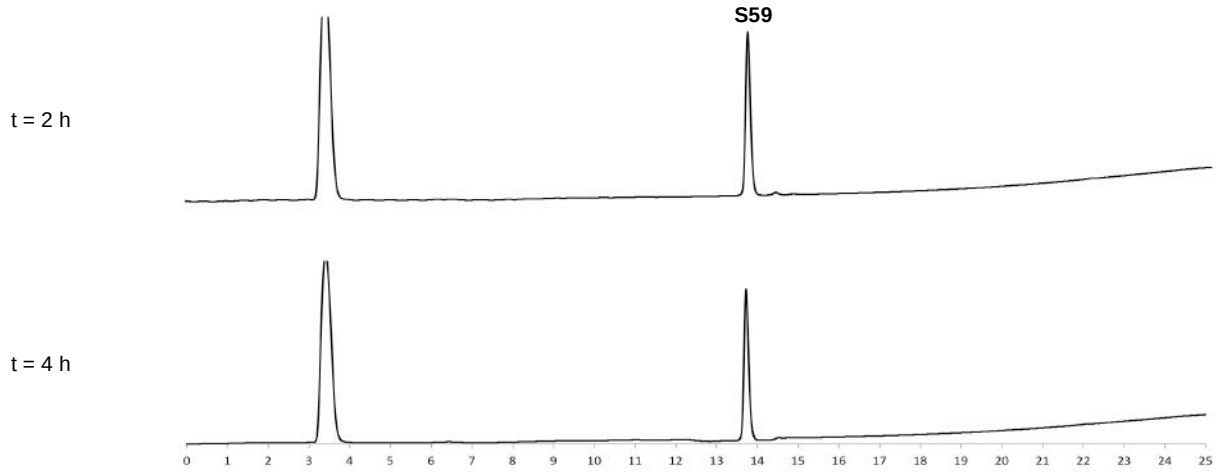
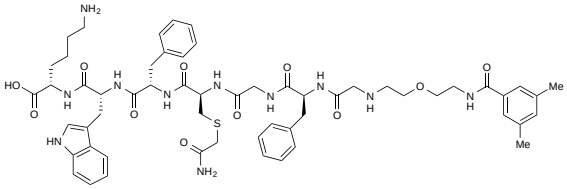


Fig. S149. Trypsin assay of **B2**

16.4.2. Characterization of degradation products

Degradation product **S59**



HRMS (MALDI) calcd for $C_{57}H_{74}N_{11}O_{11}S$ $[M+H]^+$: 1120.5284, found: 1120.5282.

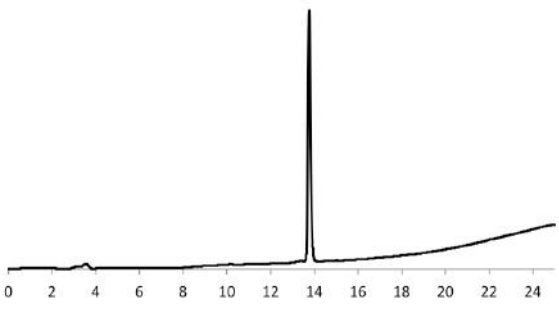


Fig. S150. Analytical HPLC of purified **S59**

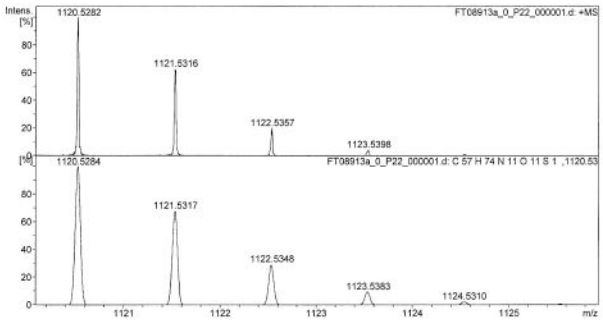


Fig. S151. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **S59**

16.5. Lasso peptide L3

16.5.1. Analytical HPLC traces

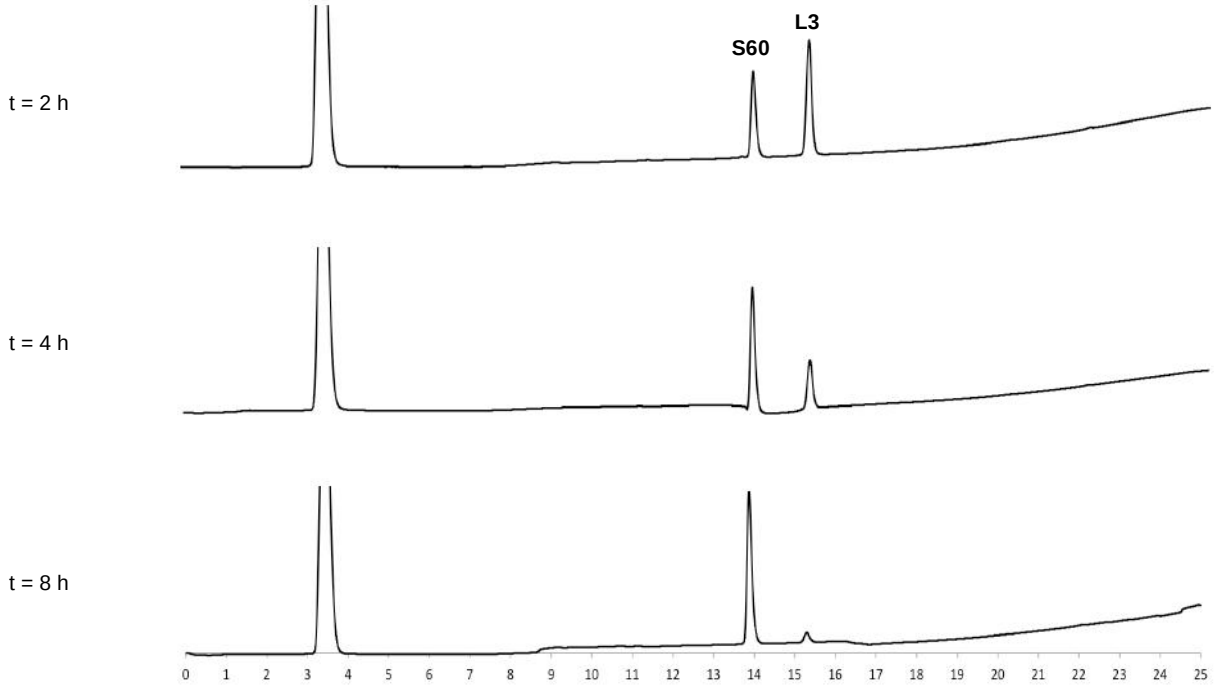
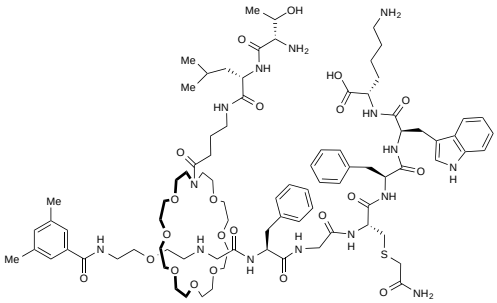


Fig. S152. Trypsin assay of **L3**

16.5.2. Characterization of degradation products

Degradation product **S60** (Shown here is a possible structure based on specificity of trypsin)



HRMS (MALDI) calcd for $C_{87}H_{132}N_{15}O_{22}S$ $[M+H]^+$: 1770.9387, found: 1770.9372.

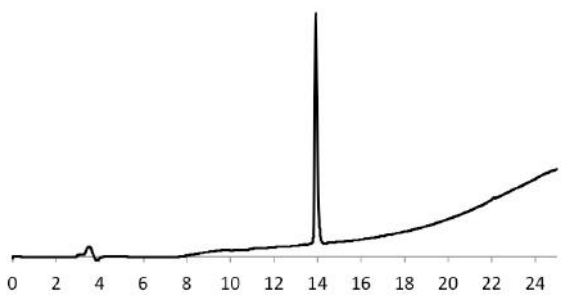


Fig. S153. Analytical HPLC of purified **S60**

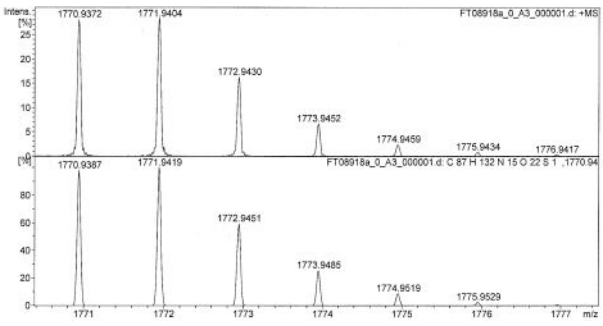


Fig. S154. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **S60**

16.6. Branched-cyclic peptide B3

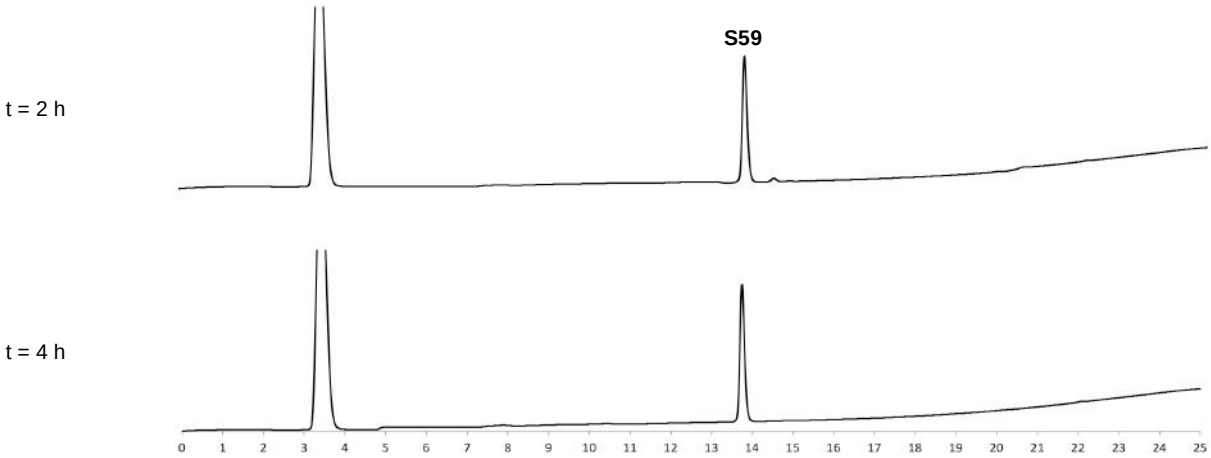


Fig. S155. Trypsin assay of **B3**

16.7. Cyclic peptide C1

16.7.1. Analytical HPLC traces

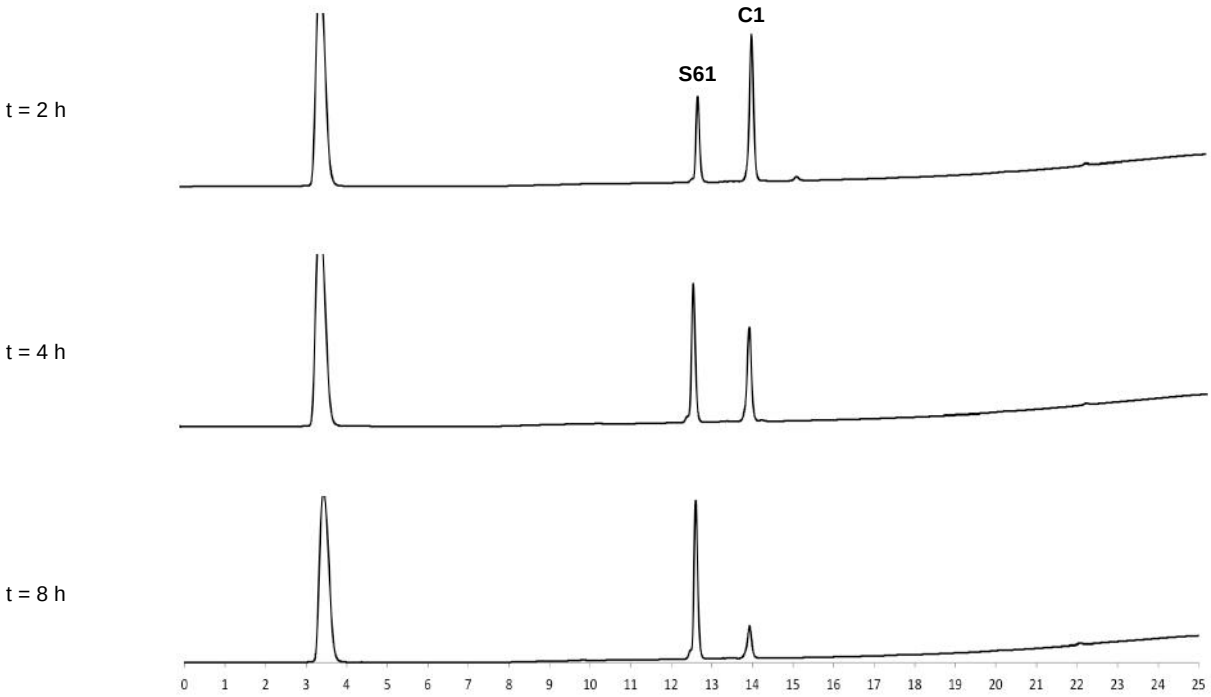
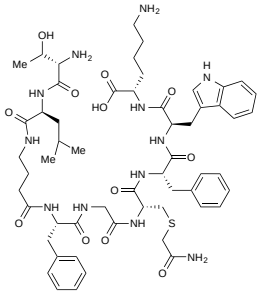


Fig. S156. Trypsin assay of **C1**

16.7.2. Characterization of degradation products

Degradation product **S61** (Shown here is a possible structure based on specificity of trypsin)



HRMS (MALDI) calcd for $C_{56}H_{79}N_{12}O_{12}S$ $[M+H]^+$: 1143.5656, found: 1143.5656.

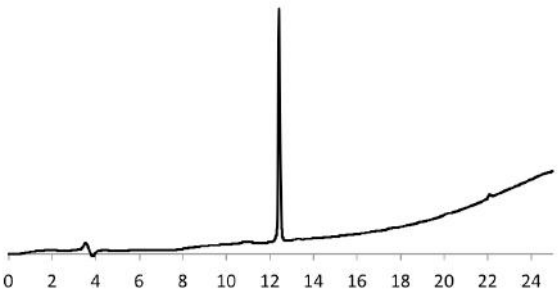


Fig. S157. Analytical HPLC of purified **S61**

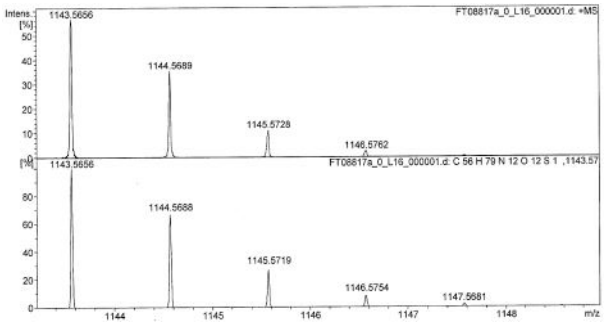


Fig. S158. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **S61**

16.8. Plots of time vs conversion

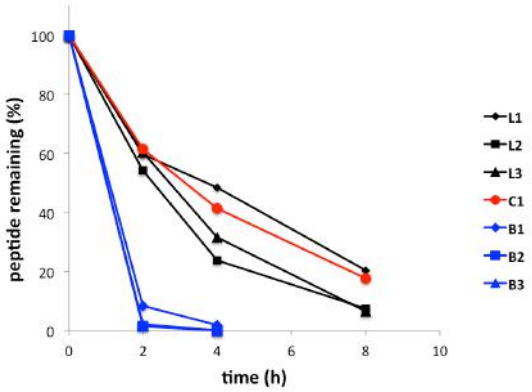


Fig. S159. Plots of time vs conversion (trypsin assay)

17. Proteinase K assay

A solution of 20 μg of peptide was diluted with 51 μL of a buffer (50 mM Tris-HCl, pH 7.6). An aliquot (10 μL) was taken from this solution, diluted with H_2O/ CH_3CN (1:1, 10 μL, 0.1% TFA) and injected to analytical RP-HPLC. The peak area of the peptide was determined by integration.

A solution of 20 μg of peptide was diluted with 50 μL of a buffer (50 mM Tris-HCl, pH 7.6). To this solution was added proteinase K dissolved in the same buffer (1.0 μg/μL, 1.0 μL). The mixture was incubated at 37 °C for 8 h and analyzed at selected time points: 2, 4, and 8 h for lasso peptides and cyclic peptide; 2 and 4 h for branched-cyclic peptides. For analysis, an aliquot (10 μL) was taken from the reaction mixture, diluted with H_2O/CH_3CN (1:1, 10 μL, 0.1% TFA) and injected to analytical RP-HPLC. The peak area of the starting peptide was determined by integration. Percentage of the remaining peptide was calculated as described in ESI section 15.

17.1. Lasso peptide L1

17.1.1. Analytical HPLC traces

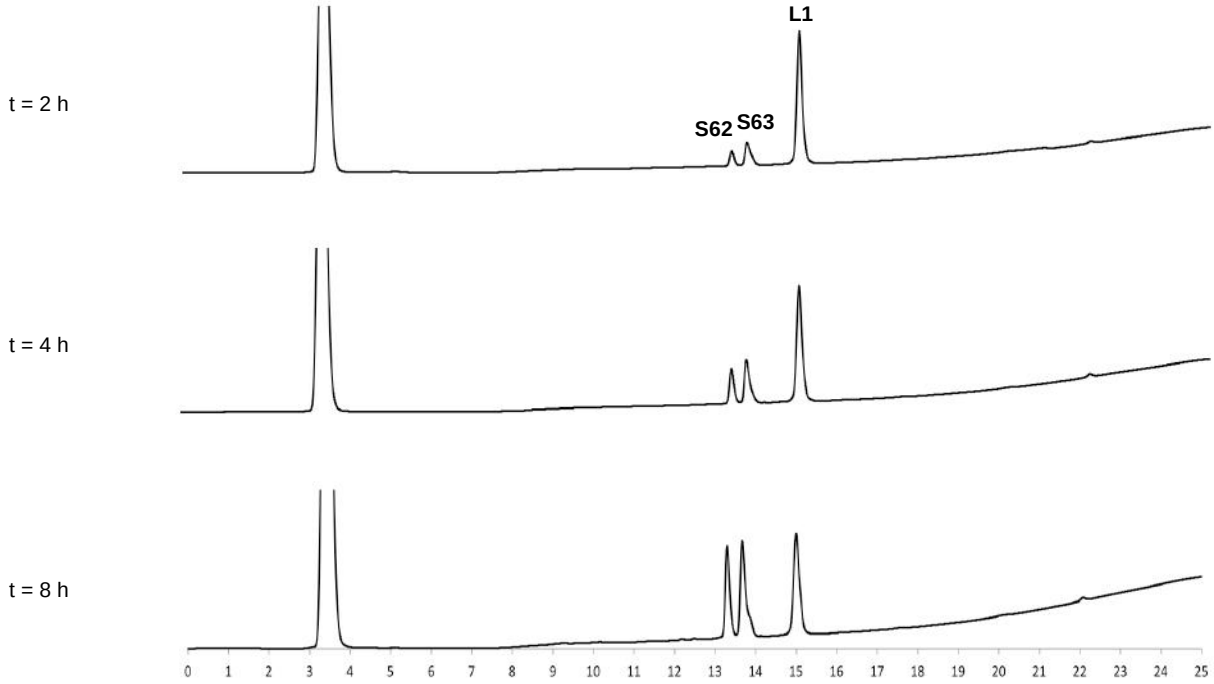
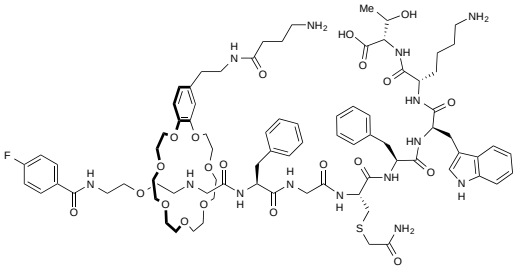


Fig. S160. Proteinase K assay of **L1**

17.1.2. Characterization of degradation products

Degradation product **S62**



HRMS (MALDI) calcd for $C_{83}H_{116}FN_{14}O_{21}S$ $[M+H]^+$: 1695.8139, found: 1695.8110.

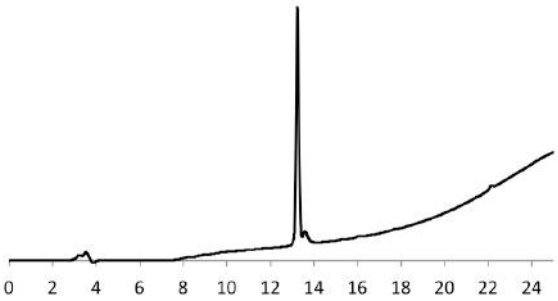


Fig. S161. Analytical HPLC of purified **S62**

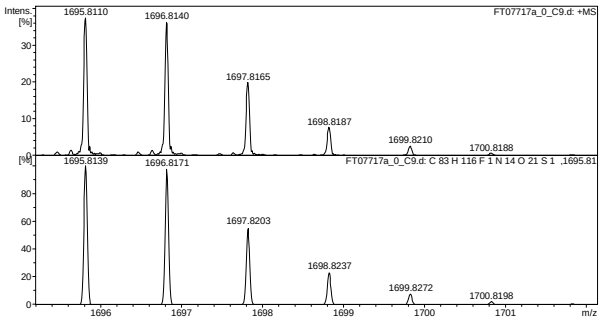
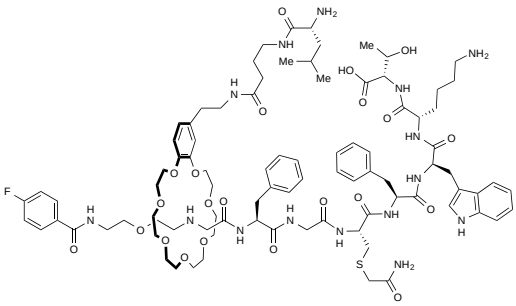


Fig. S162. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **S62**

Degradation product **S63** (Shown here is a possible structure based on specificity of proteinase K)



HRMS (MALDI) calcd for $C_{89}H_{127}FN_{15}O_{22}S$ $[M+H]^+$: 1808.8979, found: 1808.8987.

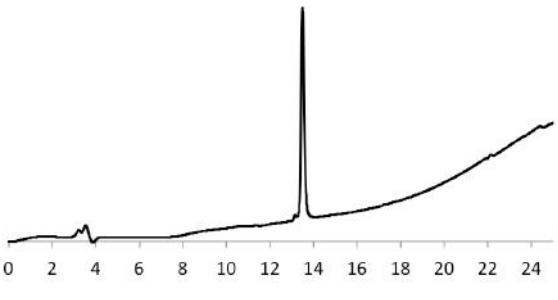


Fig. S163. Analytical HPLC of purified **S63**

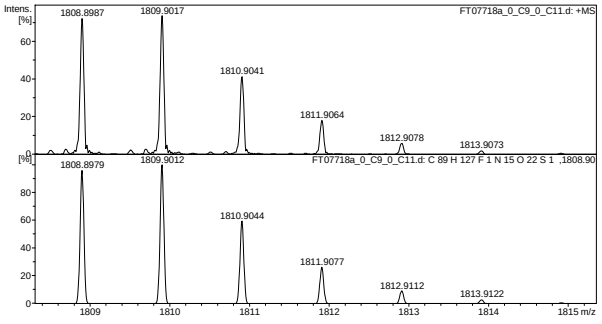


Fig. S164. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **S63**

17.2. Branched-cyclic peptide B1

17.2.1. Analytical HPLC traces

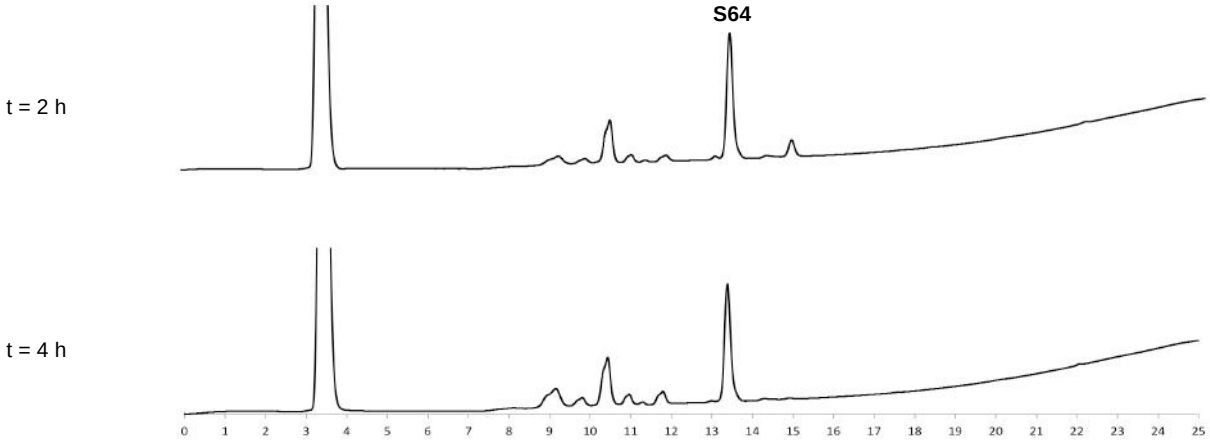
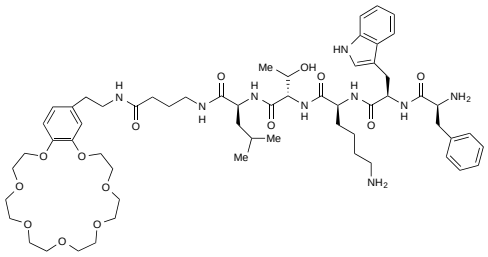


Fig. S165. Proteinase K assay of **B1**

17.2.2. Characterization of degradation products

Degradation product **S64**



HRMS (MALDI) calcd for $C_{60}H_{90}N_9O_{14}$ $[M+H]^+$: 1160.6602, found: 1160.6602.

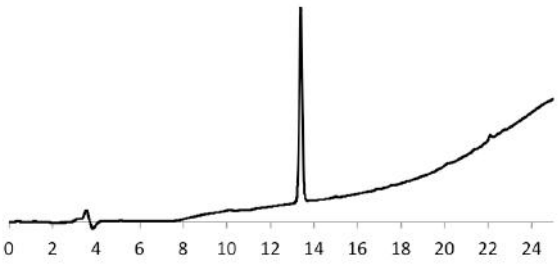


Fig. S166. Analytical HPLC of purified **S64**

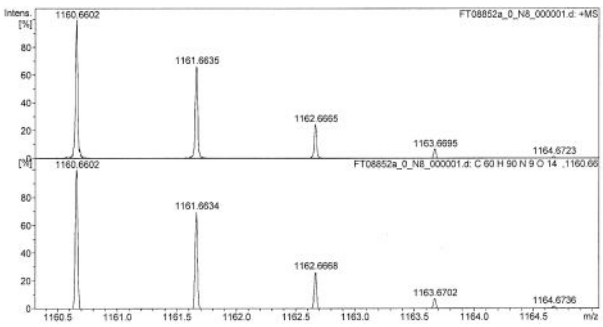


Fig. S167. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **S64**

17.3. Lasso peptide L2

17.3.1. Analytical HPLC traces

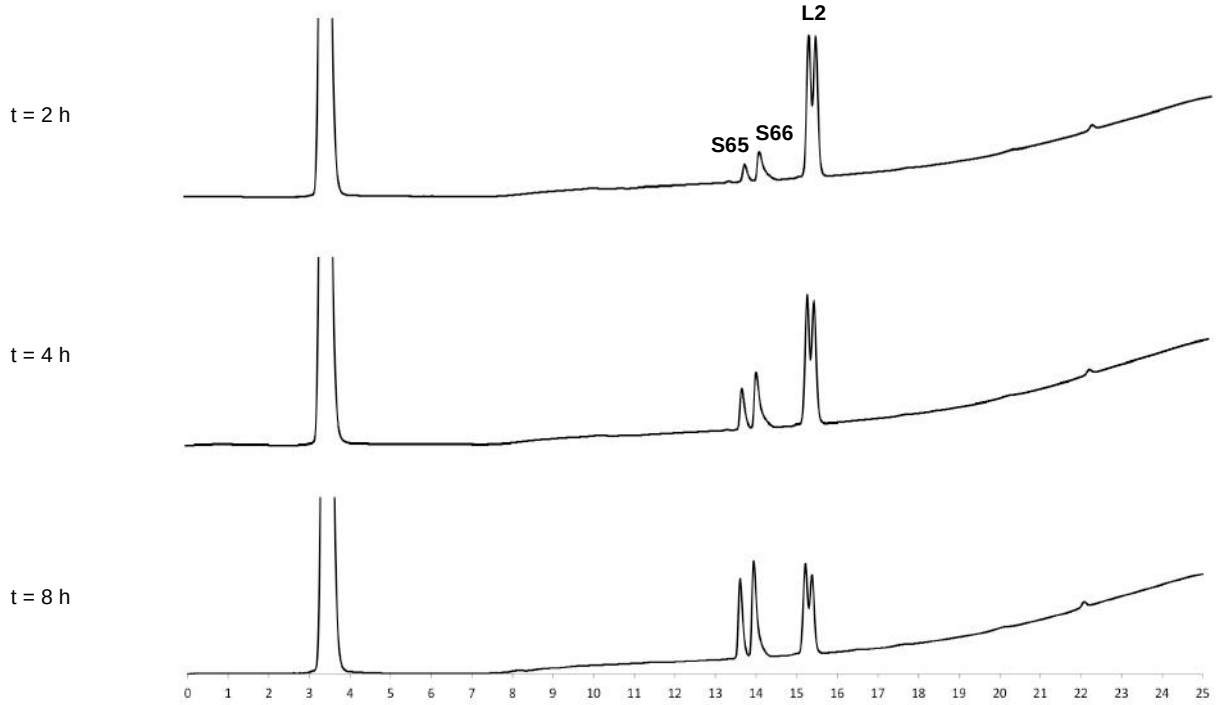
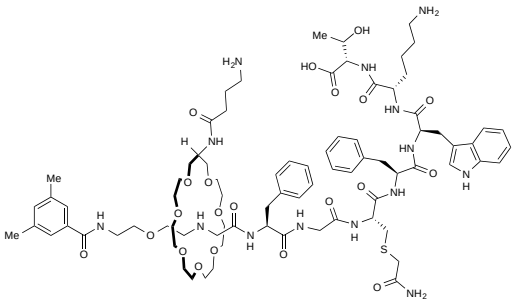


Fig. S168. Proteinase K assay of **L2**

17.3.2. Characterization of degradation products

Degradation product **S65**



HRMS (MALDI) calcd for $C_{80}H_{119}N_{14}O_{21}S$ $[M+H]^+$: 1643.8389, found: 1643.8392.

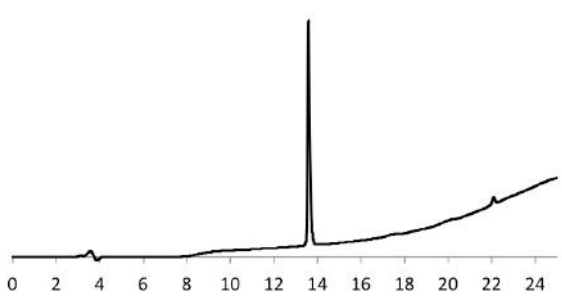


Fig. S169. Analytical HPLC of purified **S65**

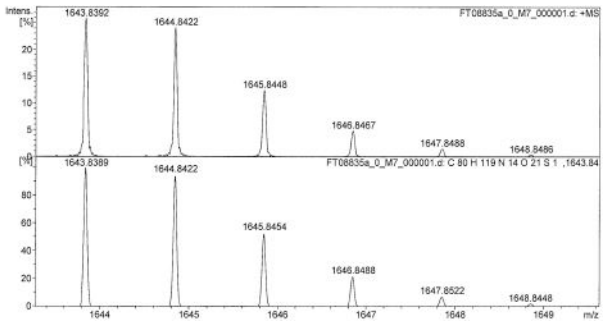
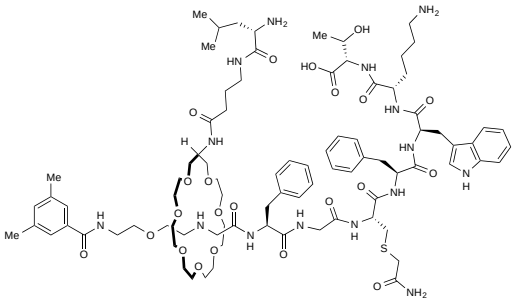


Fig. S170. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **S65**

Degradation product **S66** (Shown here is a possible structure based on specificity of proteinase K)



HRMS (MALDI) calcd for $C_{86}H_{129}N_{15}NaO_{22}S$ $[M+Na]^+$: 1778.9050, found: 1778.9042.

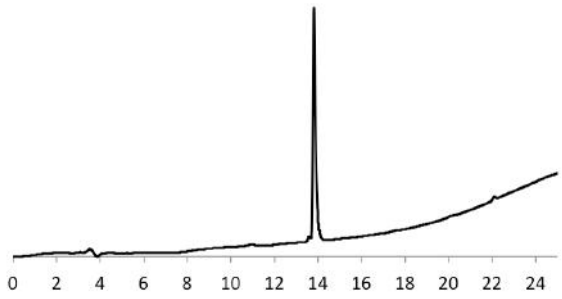


Fig. S171. Analytical HPLC of purified **S66**

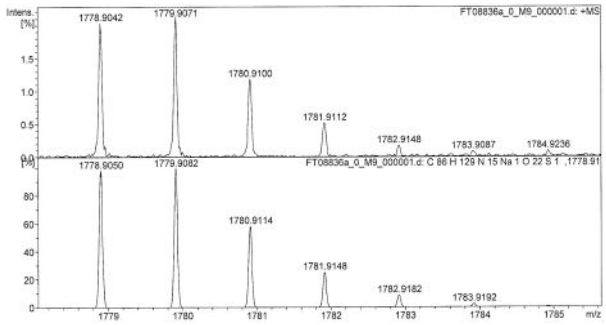


Fig. S172. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **S66**

17.4. Branched-cyclic peptide B2

17.4.1. Analytical HPLC traces

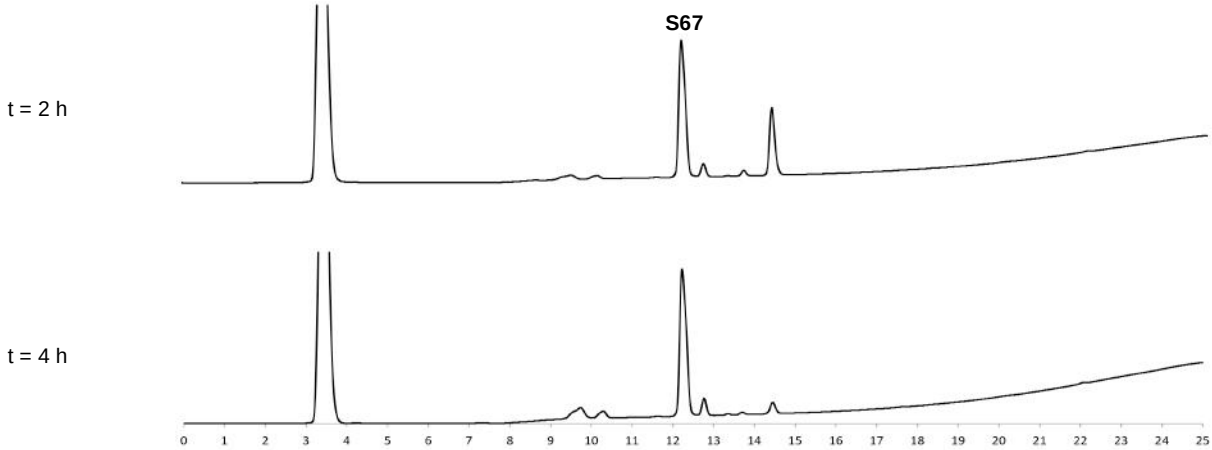
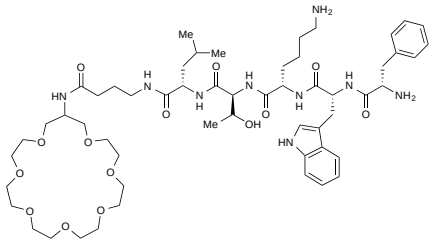


Fig. S173. Proteinase K assay of **B2**

17.4.2. Characterization of degradation products

Degradation product **S67**



HRMS (MALDI) calcd for $C_{55}H_{88}N_9O_{14}$ $[M+H]^+$: 1098.6445, found: 1098.6444.

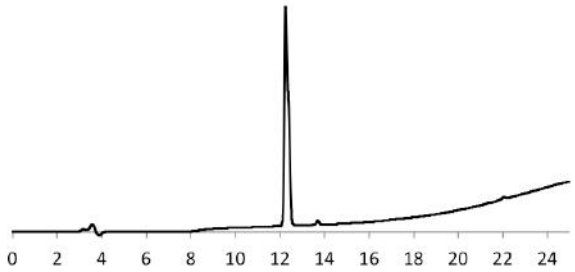


Fig. S174. Analytical HPLC of **S67** (mixture)

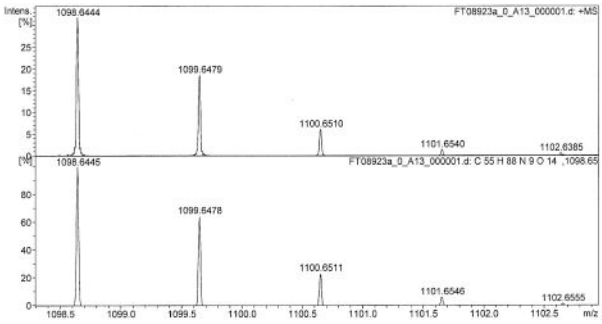


Fig. S175. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **S67**

17.5. Lasso peptide L3

17.5.1. Analytical HPLC traces

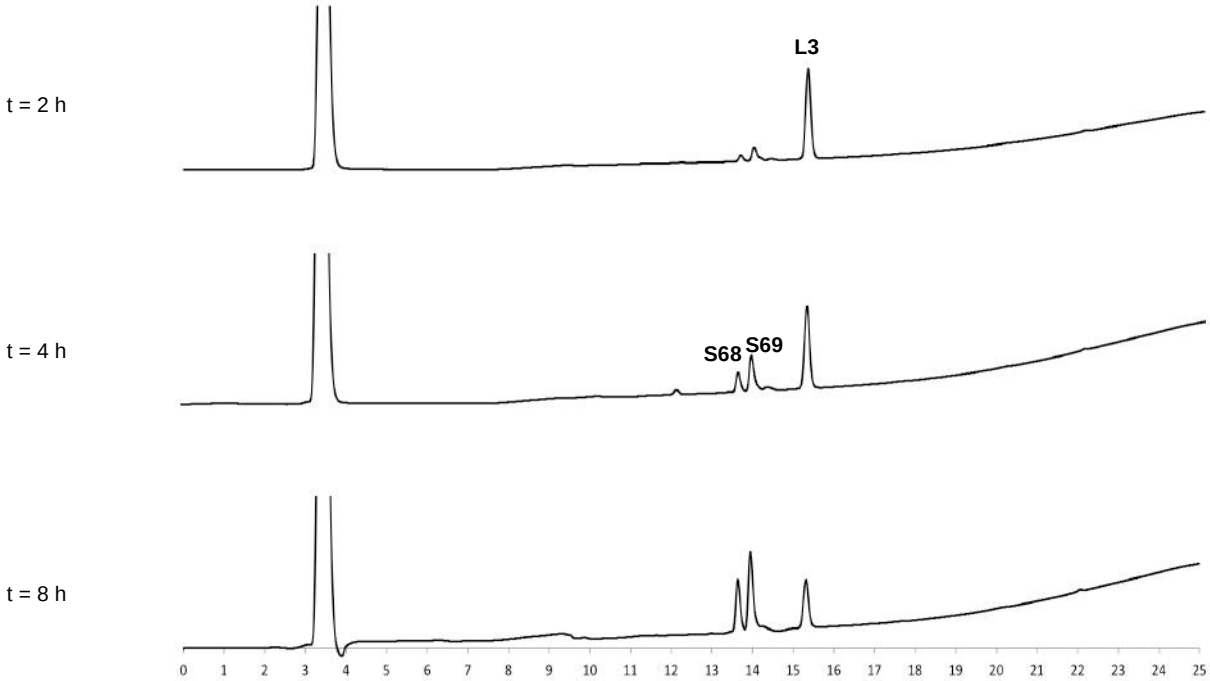
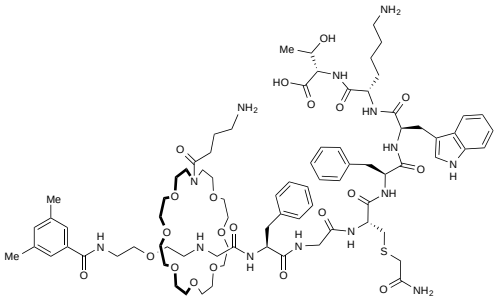


Fig. S176. Proteinase K assay of **L3**

17.5.2. Characterization of degradation products

Degradation product **S68**



HRMS (MALDI) calcd for $C_{81}H_{121}N_{14}O_{21}S$ $[M+H]^+$: 1657.8546, found: 1657.8534.

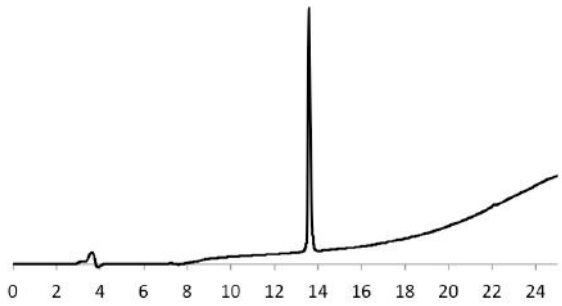


Fig. S177. Analytical HPLC of purified **S68**

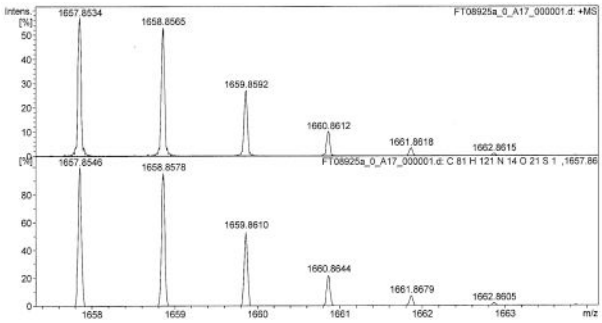
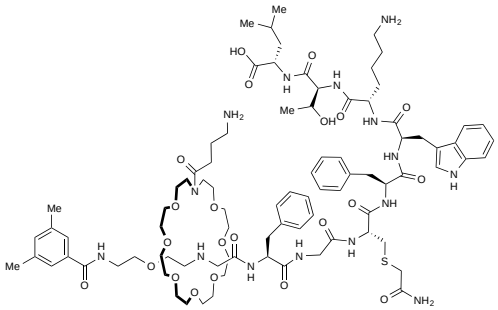


Fig. S178. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **S68**

Degradation product **S69** (Shown here is a possible structure based on specificity of proteinase K)



HRMS (MALDI) calcd for $C_{87}H_{132}N_{15}O_{22}S$ $[M+H]^+$: 1770.9387, found: 1770.9337.

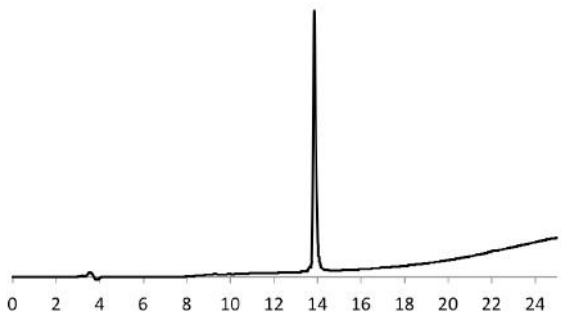


Fig. S179. Analytical HPLC of purified **S69**

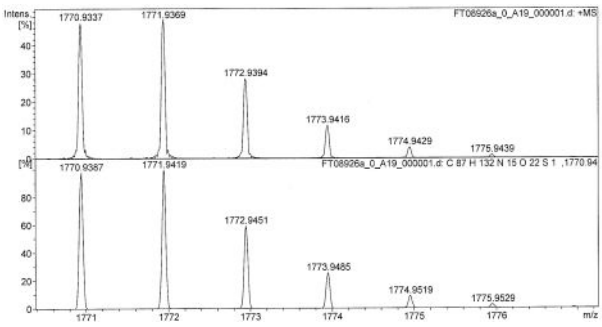


Fig. S180. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **S69**

17.6. Branched-cyclic peptide B3

17.6.1. Analytical HPLC traces

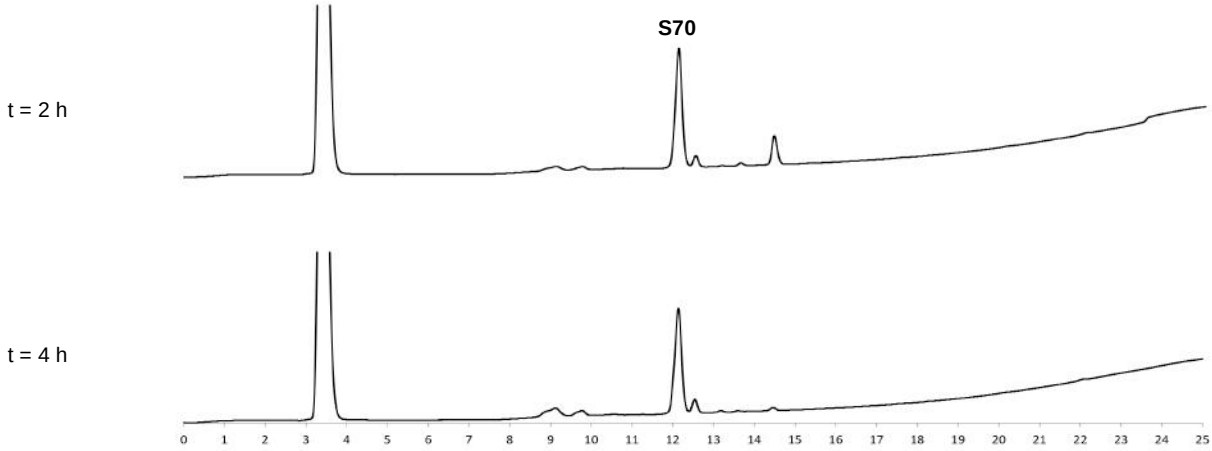
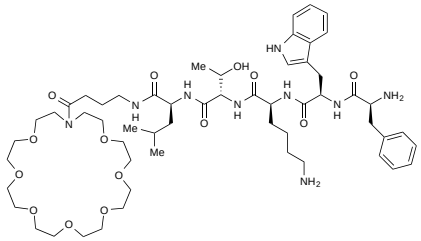


Fig. S181. Proteinase K assay of **B3**

17.6.2. Characterization of degradation products

Degradation product **S70**



HRMS (MALDI) calcd for $C_{56}H_{90}N_9O_{14}$ $[M+H]^+$: 1112.6602, found: 1112.6606.

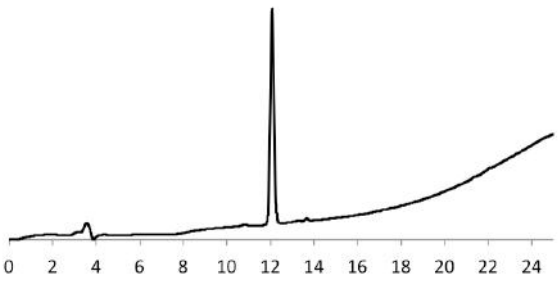


Fig. S182. Analytical HPLC of **S70** (mixture)

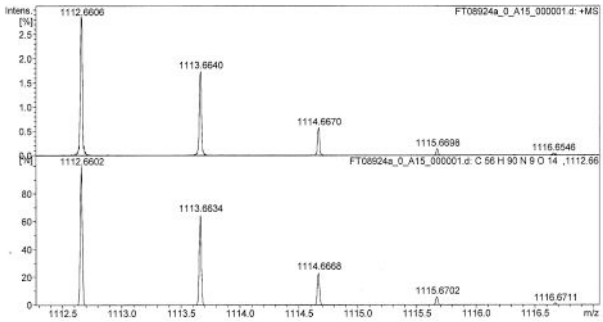


Fig. S183. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **S70**

17.7. Cyclic peptide C1

17.7.1. Analytical HPLC traces

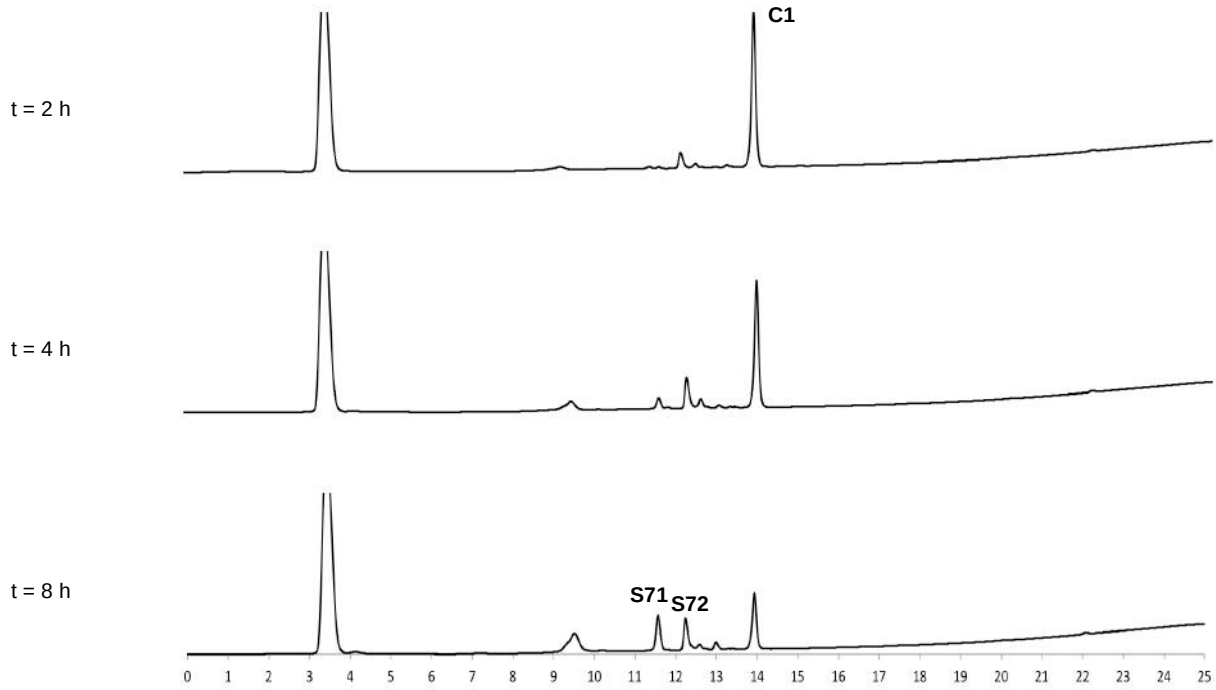
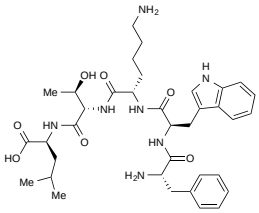


Fig. S184. Proteinase K assay of **C1**

17.7.2. Characterization of degradation products

Degradation product **S71**



HRMS (MALDI) calcd for $C_{36}H_{52}N_7O_7$ $[M+H]^+$: 694.3923, found: 694.3921.

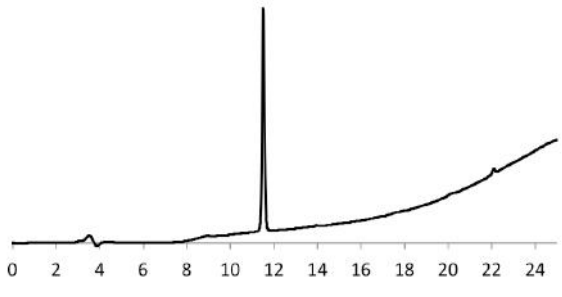


Fig. S185. Analytical HPLC of purified **S71**

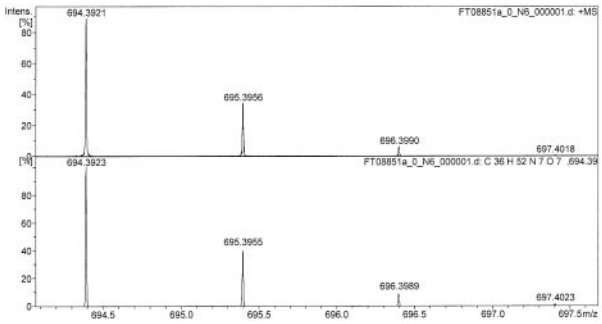
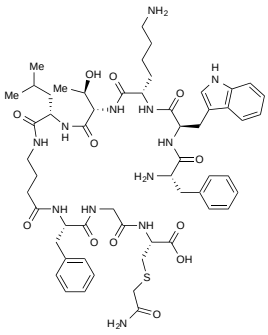


Fig. S186. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **S71**

Degradation product **S72** (Shown here is a possible structure based on specificity of proteinase K)



HRMS (MALDI) calcd for $C_{56}H_{79}N_{12}O_{12}S$ $[M+H]^+$: 1143.5656, found: 1143.5650.

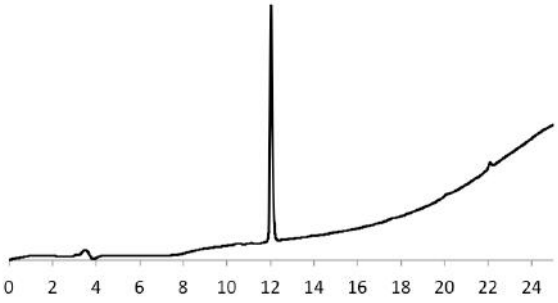


Fig. S187. Analytical HPLC of purified **S72**

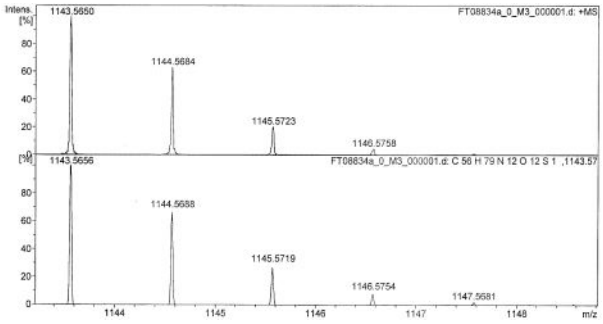


Fig. S188. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **S72**

17.8. Plots of time vs conversion

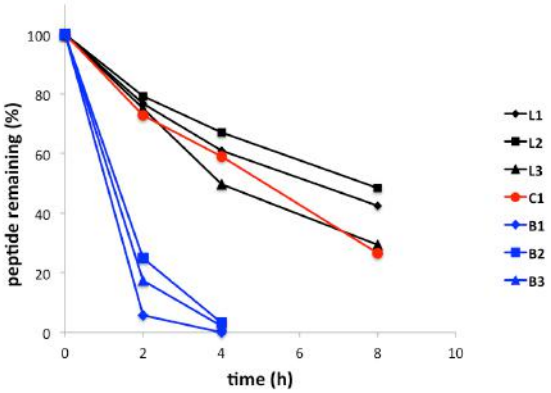
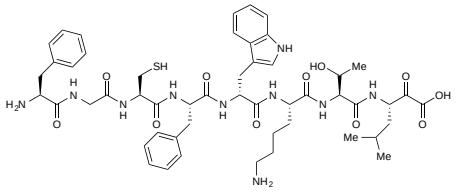


Fig. S189. Plots of time vs conversion (proteinase K assay)

18. Serum stability assay

18.1. Synthesis of linear peptide R1

18.1.1. Linear peptide α-ketoacid S73



S73

Linear peptide α-ketoacid **S73** was prepared using the protected leucine α-ketoacid resin **S1** on a 0.24 mmol scale (1.4 g) with a substitution capacity of 0.17 mmol/g. After the full assembly of amino acids, a half of the resin was transferred to another fritted syringe, and Fmoc deprotection was performed with 20% piperidine in DMF (7 min x 2). The resin was treated with (95:2.5:2.5) TFA:DODT:H_2O for 1 h and removed by filtration. The volatiles were evaporated from the filtrate under reduced pressure. The residue was triturated with Et_2O and centrifuged to obtain the crude **S73**. Purification of crude **S73** was performed by preparative HPLC using YMC C18 column (20 x 250 mm) with a gradient of 20 to 95% CH_3CN with 0.1% TFA in 28 min. The pure product fractions were pooled and lyophilized to obtain 25.2 mg of **S73** (20% yield for peptide synthesis, resin cleavage and purification steps). Analytical HPLC and HRMS were used to confirm the purity and exact mass of the product.

HRMS (MALDI) calcd for $C_{51}H_{69}N_{10}O_{11}S$ $[M+H]^+$: 1029.4863, found: 1029.4863.

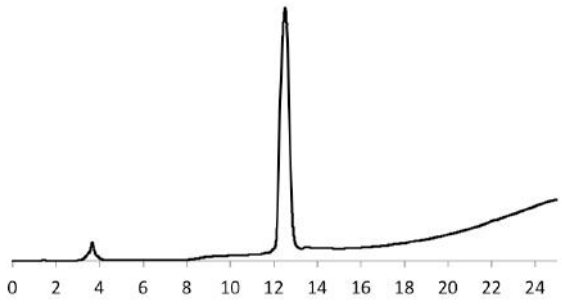


Fig. S190. Analytical HPLC of purified **S73**

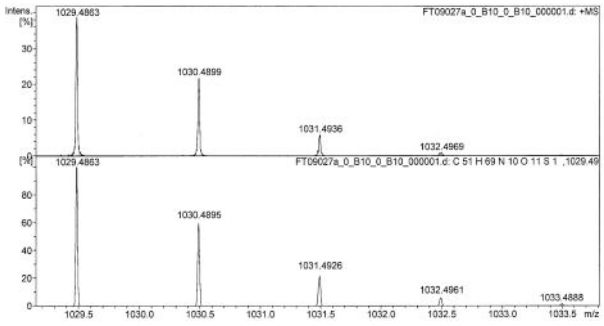
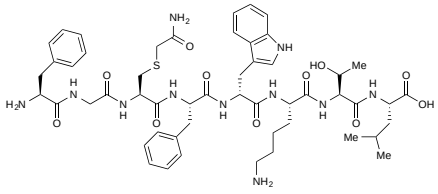


Fig. S191. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **S73**

18.1.2. Linear peptide R1



R1

To a solution of **S73** (11 mg, 10 μmol, 1.0 equiv) in 0.1 M sodium phosphate buffer (pH

7.4)/CH_3CN (1:1, 0.79 mL) was added a solution of iodoacetamide (2.1 mg, 11 μmol, 1.1 equiv) in 0.1 M sodium phosphate buffer (pH 7.4)/CH_3CN (1:1, 0.21 mL). The mixture was incubated at RT for 30 min, and 30% H_2O_2 in H_2O (0.82 mL) was added. The mixture was further incubated at RT for 5 min and immediately purified by preparative HPLC using YMC C18 column (20 x 250 mm) with a gradient of 20 to 95% CH_3CN with 0.1% TFA in 28 min. The pure product fractions were pooled and lyophilized to obtain 5.3 mg of **R1** (49% yield). Analytical HPLC and HRMS were used to confirm the purity and exact mass of the product.

HRMS (MALDI) calcd for $C_{52}H_{71}N_{11}NaO_{11}S$ $[M+Na]^+$: 1080.4947, found: 1080.4946.

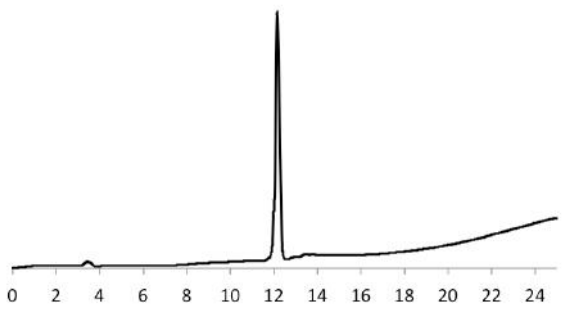


Fig. S192. Analytical HPLC of purified **R1**

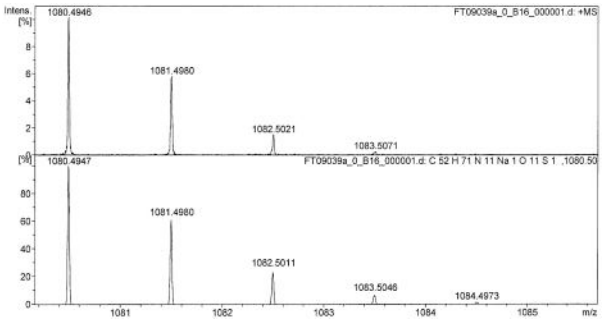


Fig. S193. HRMS (MALDI) of measured (top) and calculated (bottom) isotopic pattern of **R1**

18.2. Procedure for serum stability assay[14]

Serum stability was studied using male human serum type AB (Sigma). A 10 mM peptide solution (2.7 μL) was added to serum (132 μL) and incubated at 37 °C and analyzed at selected time points: 0 min, 2, 4, 8, and 24 h for all the peptides except linear peptide **R1**; 0 min, 2, 4, and 8 h for linear peptide **R1**. For analysis, an aliquot (15 μL) was taken from the reaction mixture and poured into cold MeOH (15 μL, pre-cooled in the freezer) to precipitate the serum proteins. The mixture was centrifuged, and an aliquot (7.5 μL) was taken from the supernatant and injected to analytical RP-HPLC. The peak area of the starting peptide was used to calculate percentage of the remaining peptide relative to the initial peptide sample (t = 0 min). Experiments were conducted in duplicate.

18.3. Plots of time vs conversion

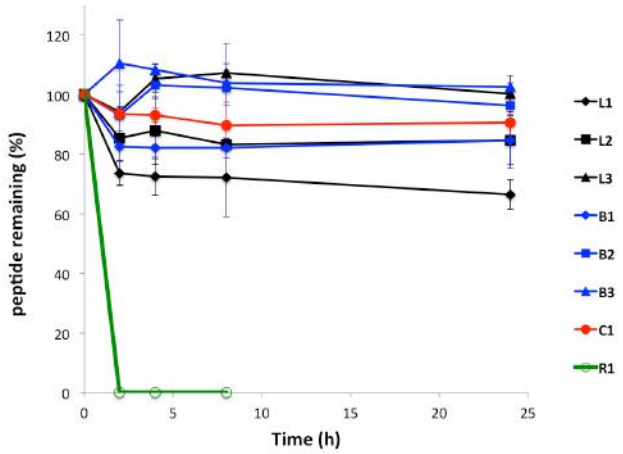


Fig. S194. Plots of time vs conversion (serum assay)

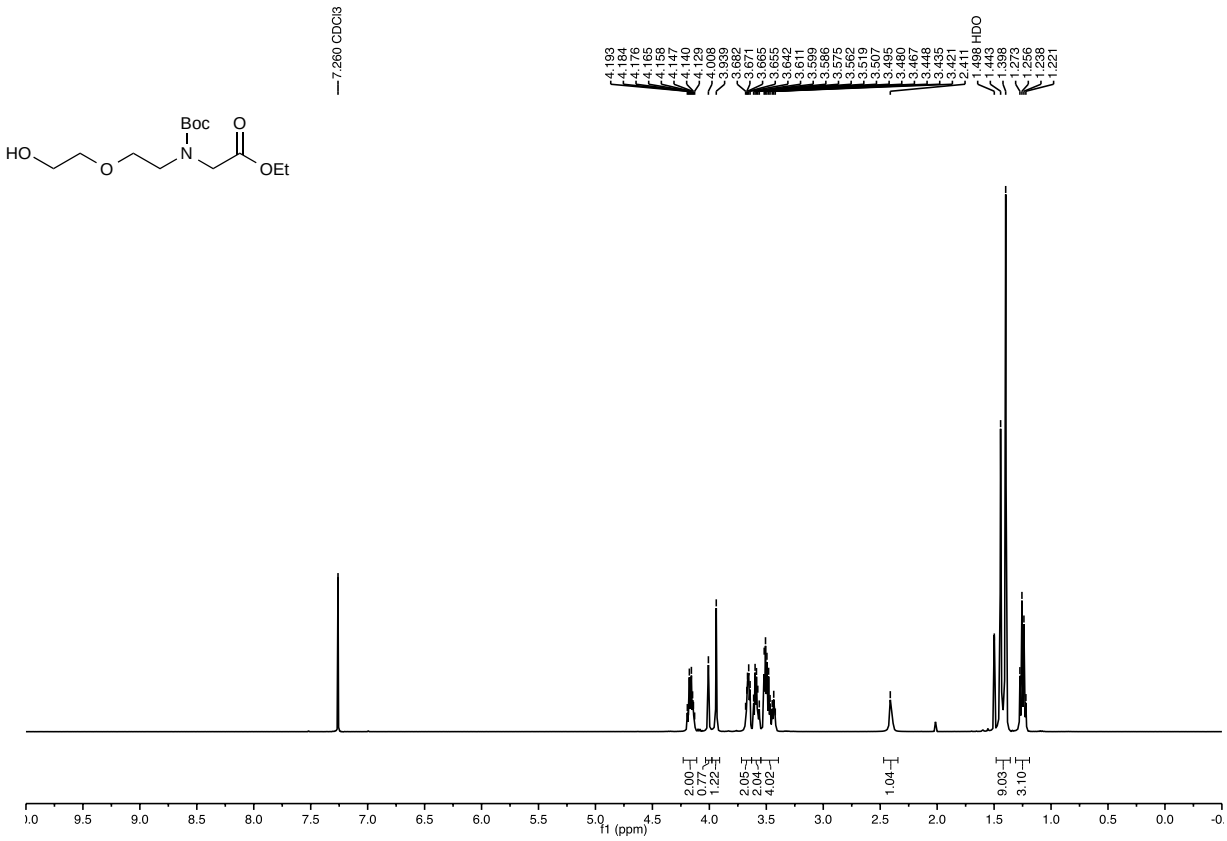
19. References

[1] Dumas, A. M.; Bode, J. W. *Org. Lett.* **2012**, *14*, 2138.
[2] Wucherpfennig, T. G.; Pattabiraman, V. R.; Limberg, F. R. P.; Ruiz-Rodríguez, J.; Bode, J. W. *Angew. Chem. Int. Ed*. **2014**, *53*, 12248.
[3] Noda, H.; Erős, G.; Bode, J. W. *J. Am. Chem. Soc.* **2014**, *136*, 5611.
[4] Zhang, C.; Li, S.; Zhang, J.; Zhu, K.; Li, N.; Huang, F. *Org. Lett.* **2007**, *9*, 5553.
[5] Dolomanov, O. V.; Bourhis, L. J.; Gildea, R. J.; Howard, J. A. K.; Puschmann, H. *J. Appl. Cryst.* **2009**, *42*, 339.
[6] Sheldrick, G. M. *Acta Cryst*. **2015**, *A71*, 3.
[7] Sheldrick, G. M. *Acta Cryst.* **2008**, *A64*, 112.
[8] Sayyadi, N.; Skropeta, D.; Jolliffe, K. A. *Org. Lett.* **2005**, *7*, 5497.
[9] Mazunin, D.; Broguiere, N.; Zenobi-Wong, M.; Bode, J. W. *ACS Biomater. Sci. Eng.* **2015**, *1*, 456.
[10] Nemoto, H.; Nishiyama, T.; Akai, S. *Org. Lett.* **2011**, *13*, 2714.
[11] Kota, R.; Samudrala, R.; Mattern, D. L. *J. Org. Chem.* **2012**, *77*, 9641.
[12] Ebner, C.; Müller, C. A.; Markert, C.; Pfaltz, A. *J. Am. Chem. Soc.* **2011**, *133*, 4710.
[13] Mao, H.; Thorne, J. B.; Pharr, J. S.; Gawley, R. E. *Can. J. Chem.* **2006**, *84*, 1273.
[14] Knappe, T. A.; Manzenrieder, F.; Mas-Moruno, C.; Linne, U.; Sasse, F.; Kessler, H.; Xie, X.; Marahiel, M. A. *Angew. Chem. Int. Ed.* **2011**, *50*, 8714.

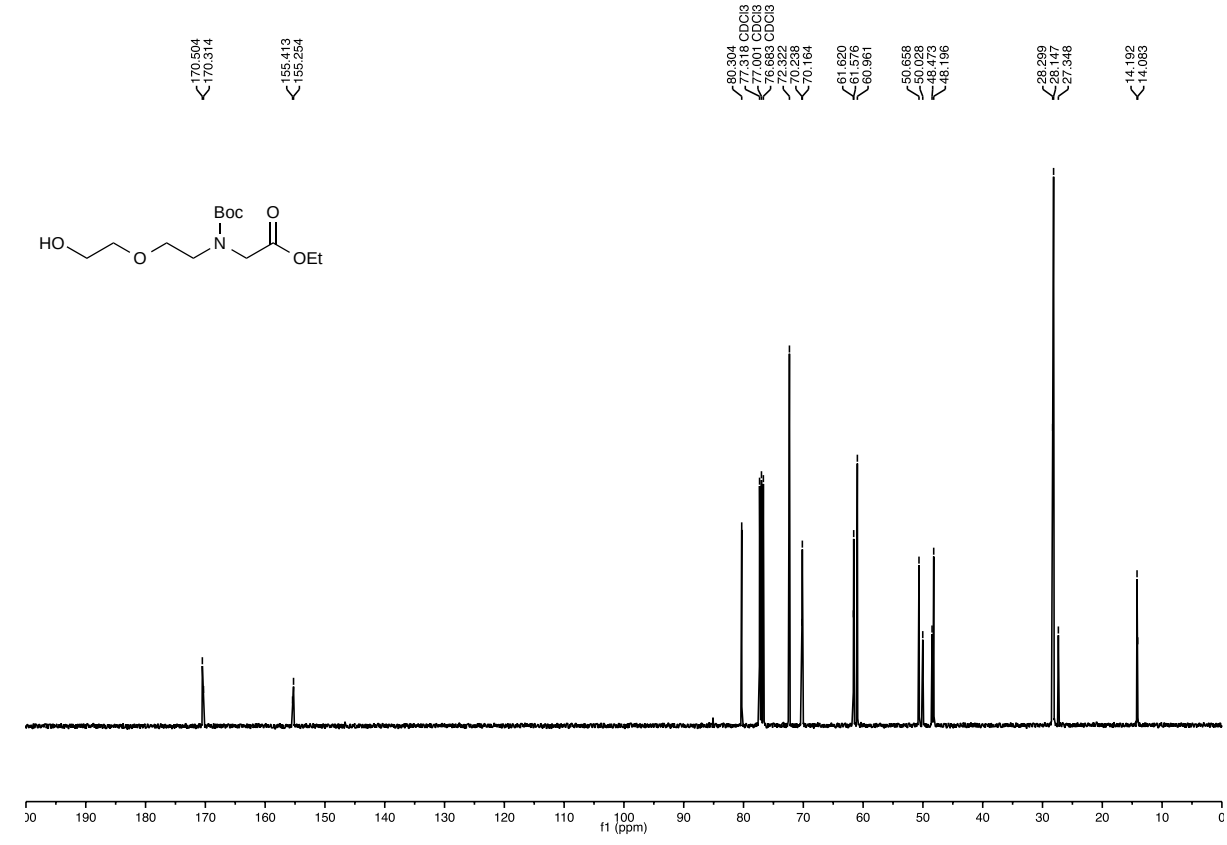
20. NMR spectra and HRMS data

Alcohol S2

^{1}H NMR (400 MHz, $CDCl_3$)

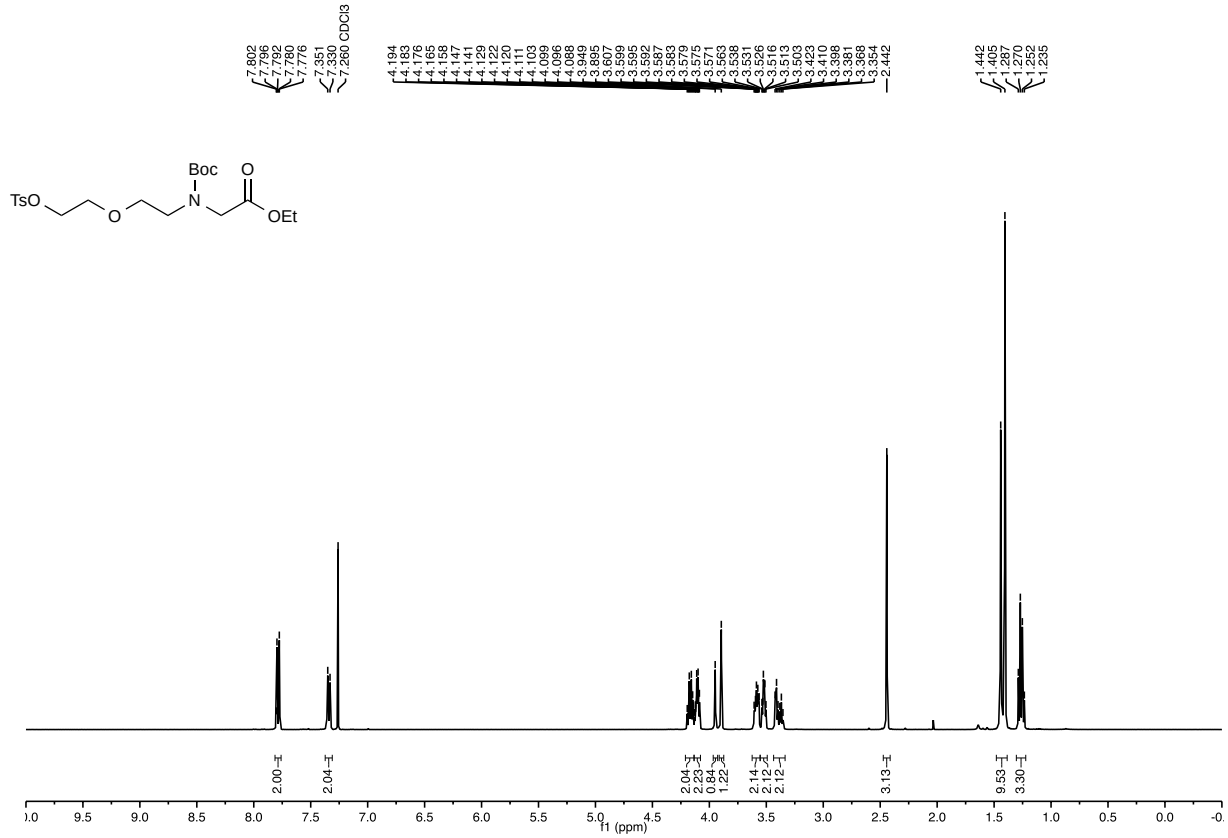


^{13}C NMR (100 MHz, $CDCl_3$)

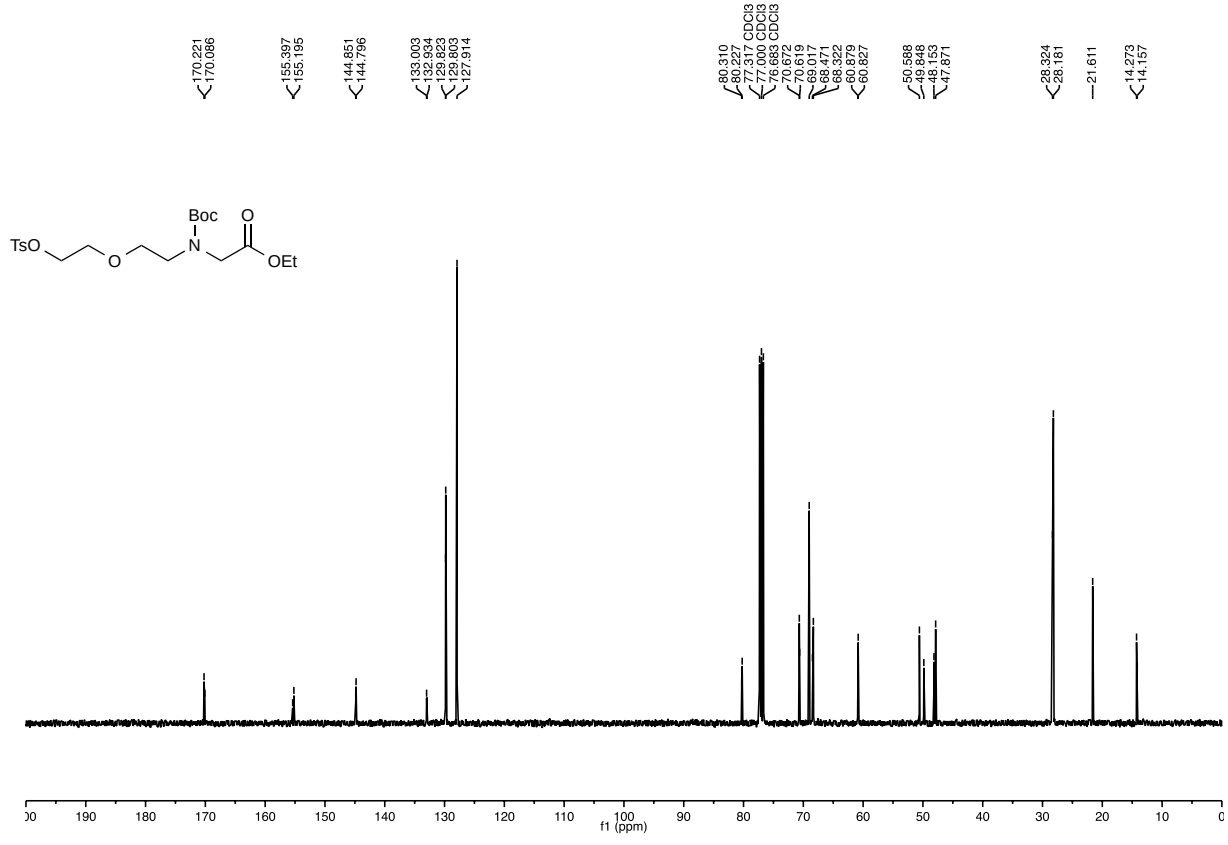


Tosylate S3

^{1}H NMR (400 MHz, $CDCl_3$)

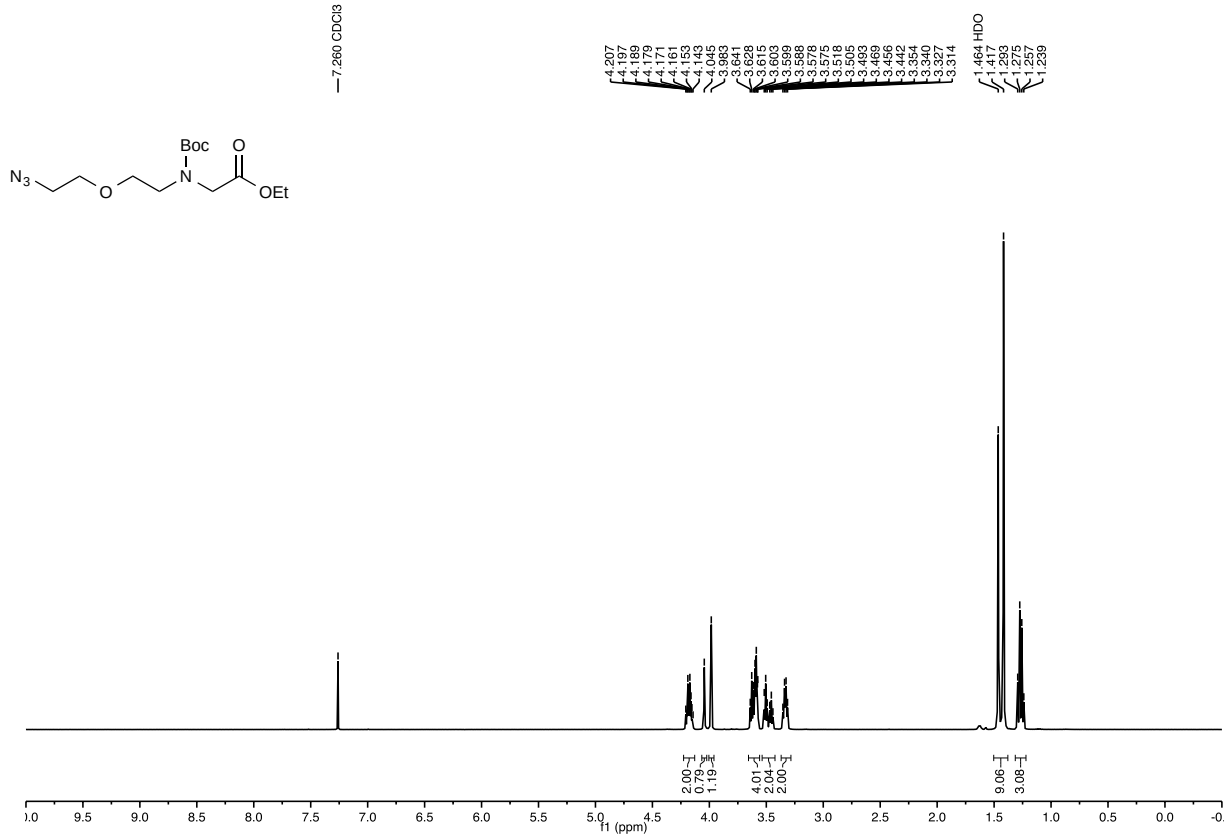


^{13}C NMR (100 MHz, $CDCl_3$)

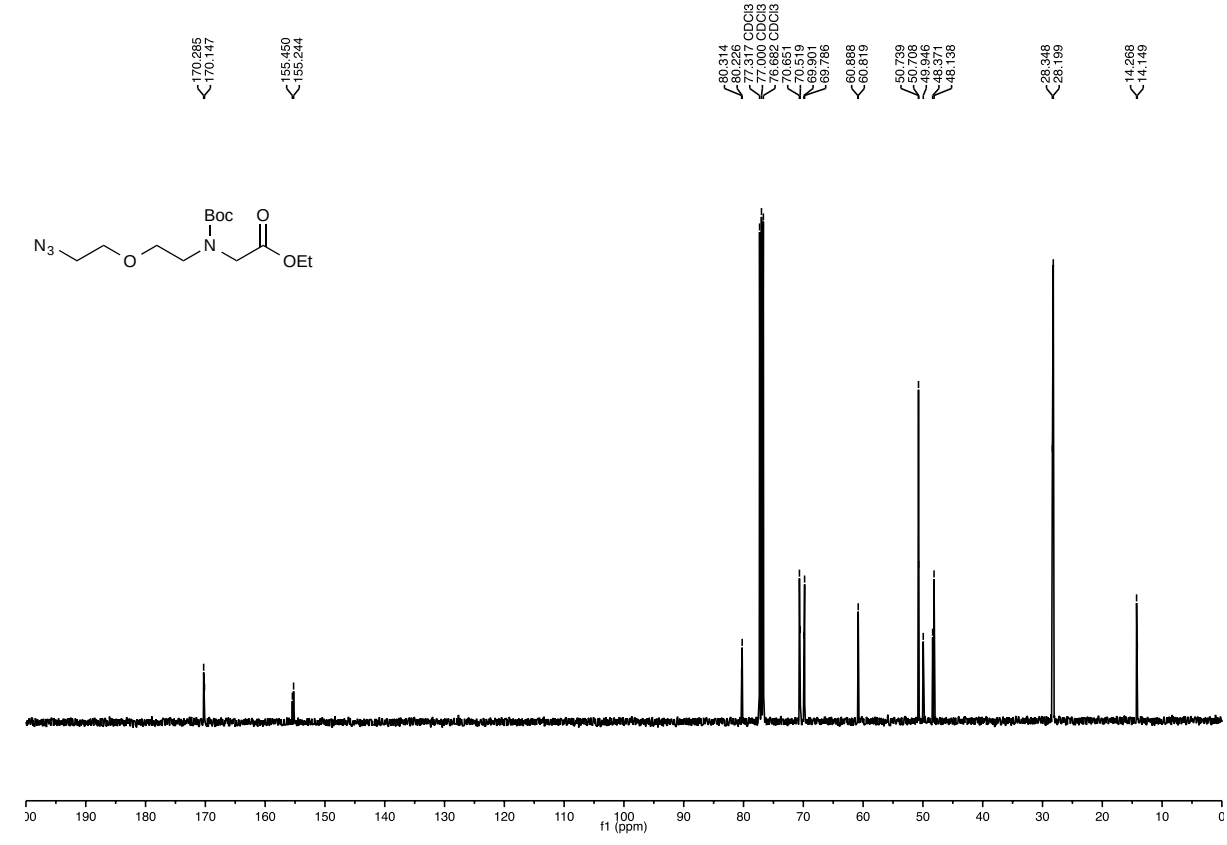


Azide-ethyl ester S4

^{1}H NMR (400 MHz, $CDCl_3$)

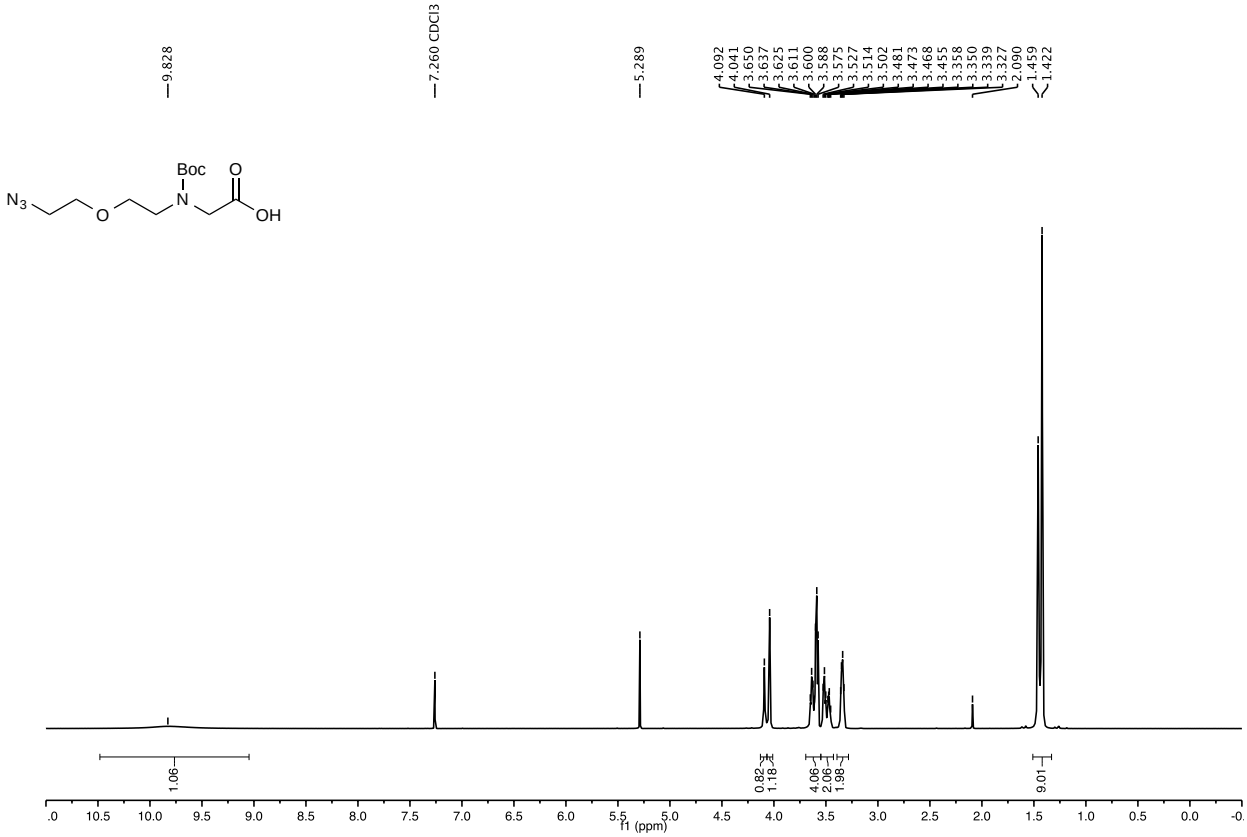


^{13}C NMR (100 MHz, $CDCl_3$)

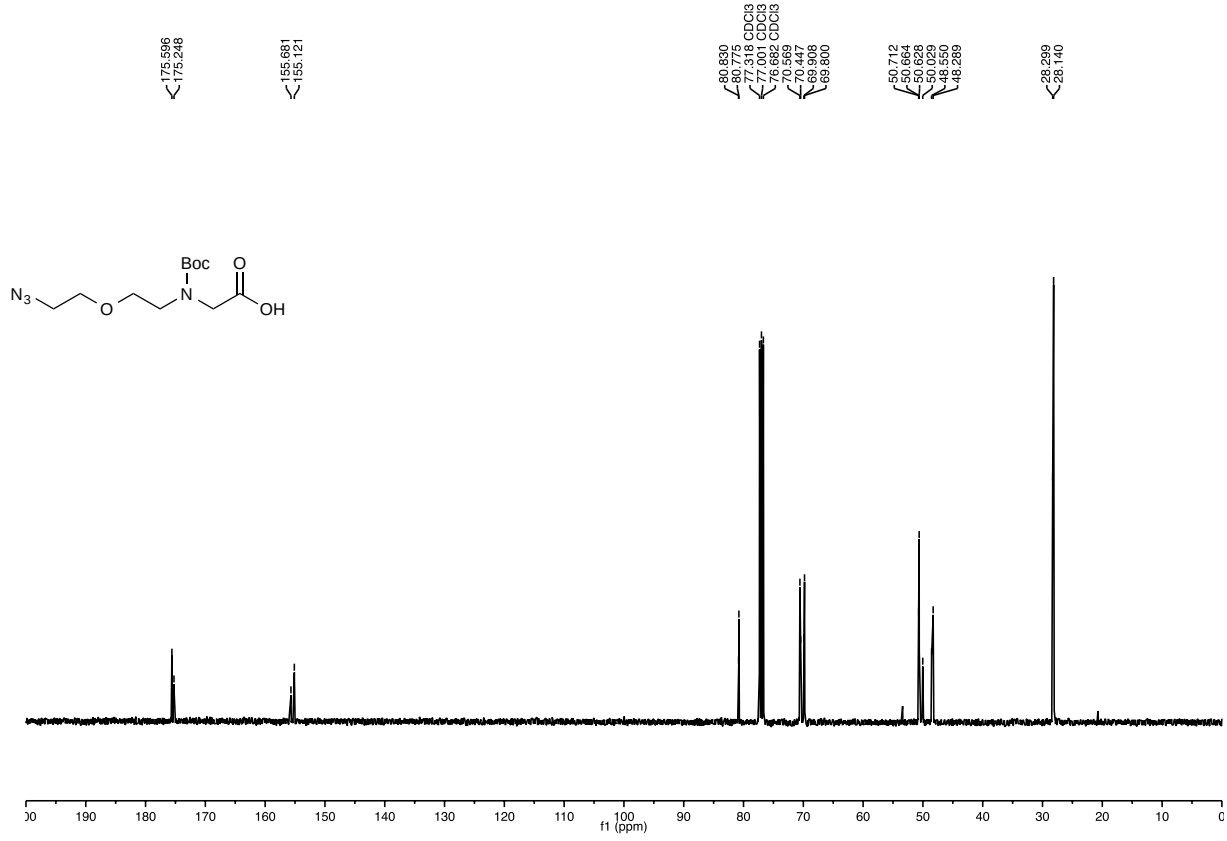


Azide-acid S5

^{1}H NMR (400 MHz, $CDCl_3$)

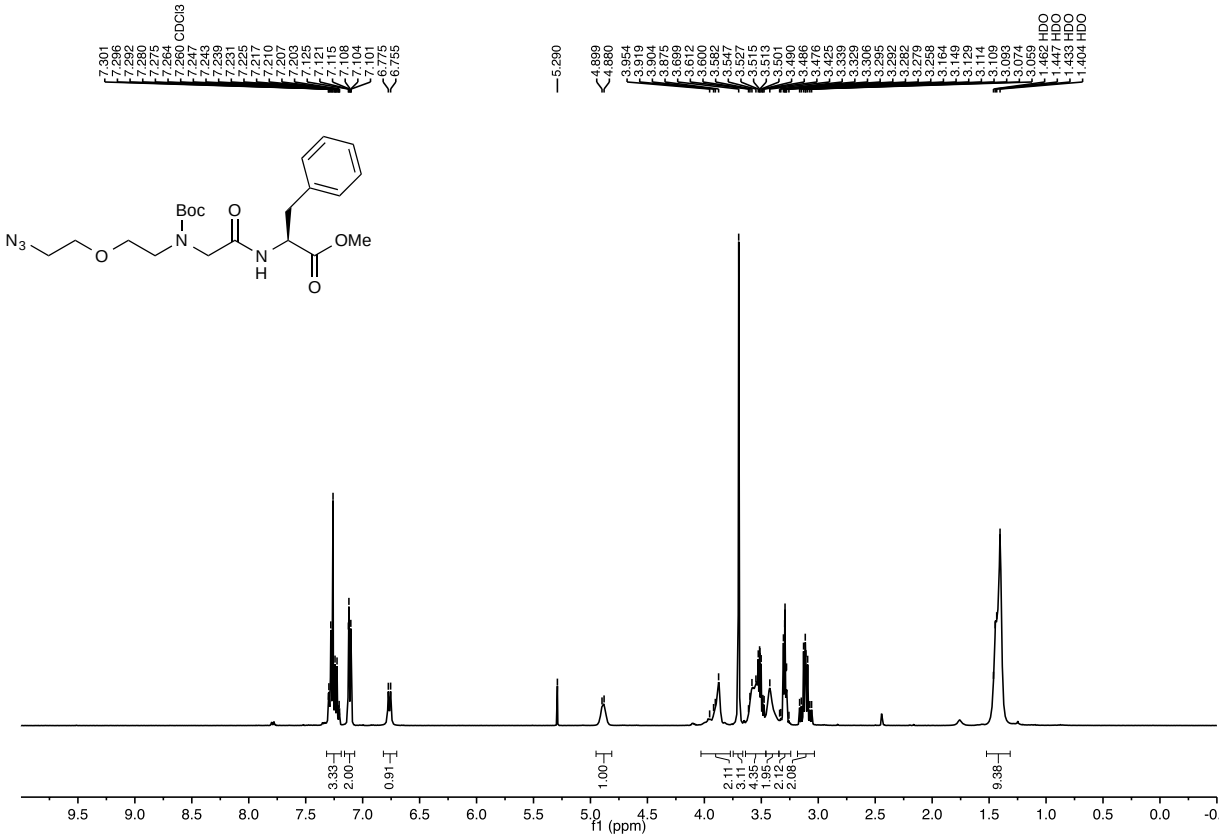


^{13}C NMR (100 MHz, $CDCl_3$)

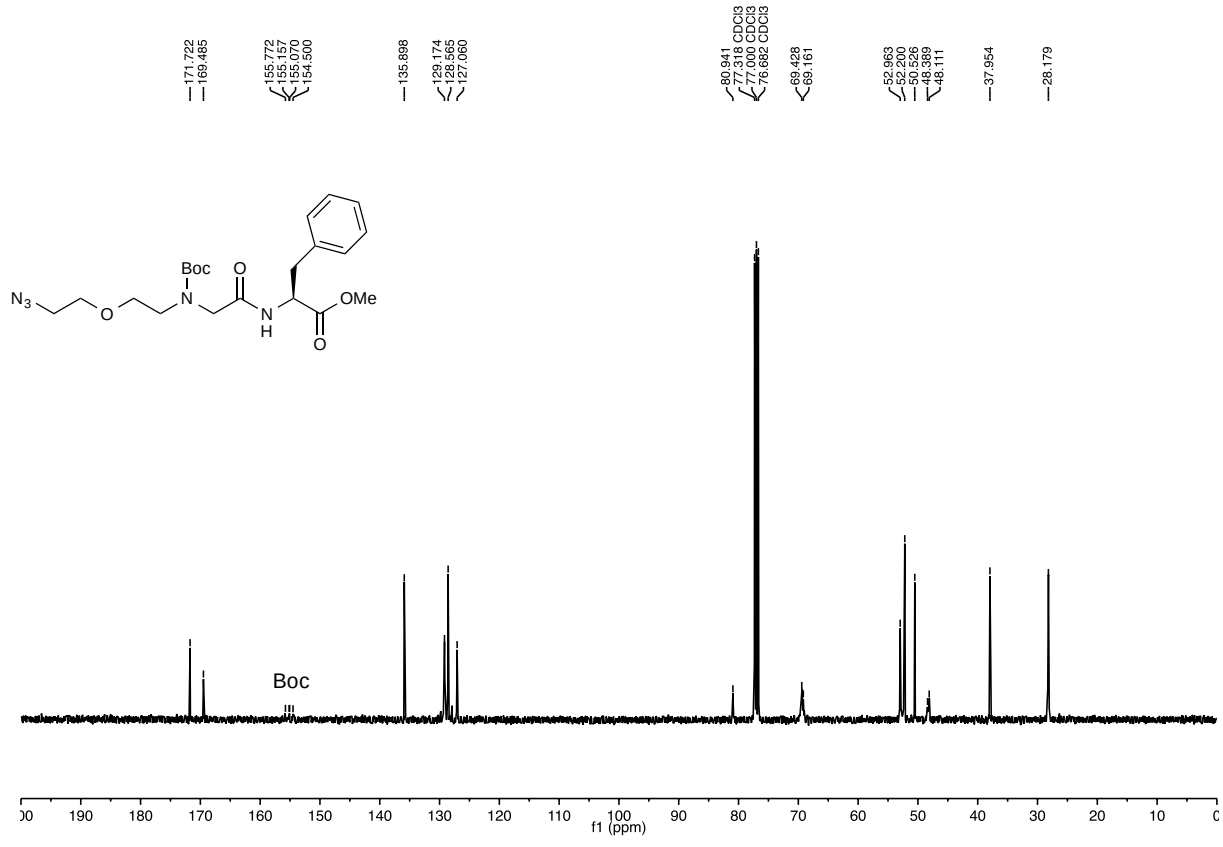


Azide-Phe methyl ester S6

^{1}H NMR (400 MHz, $CDCl_3$)

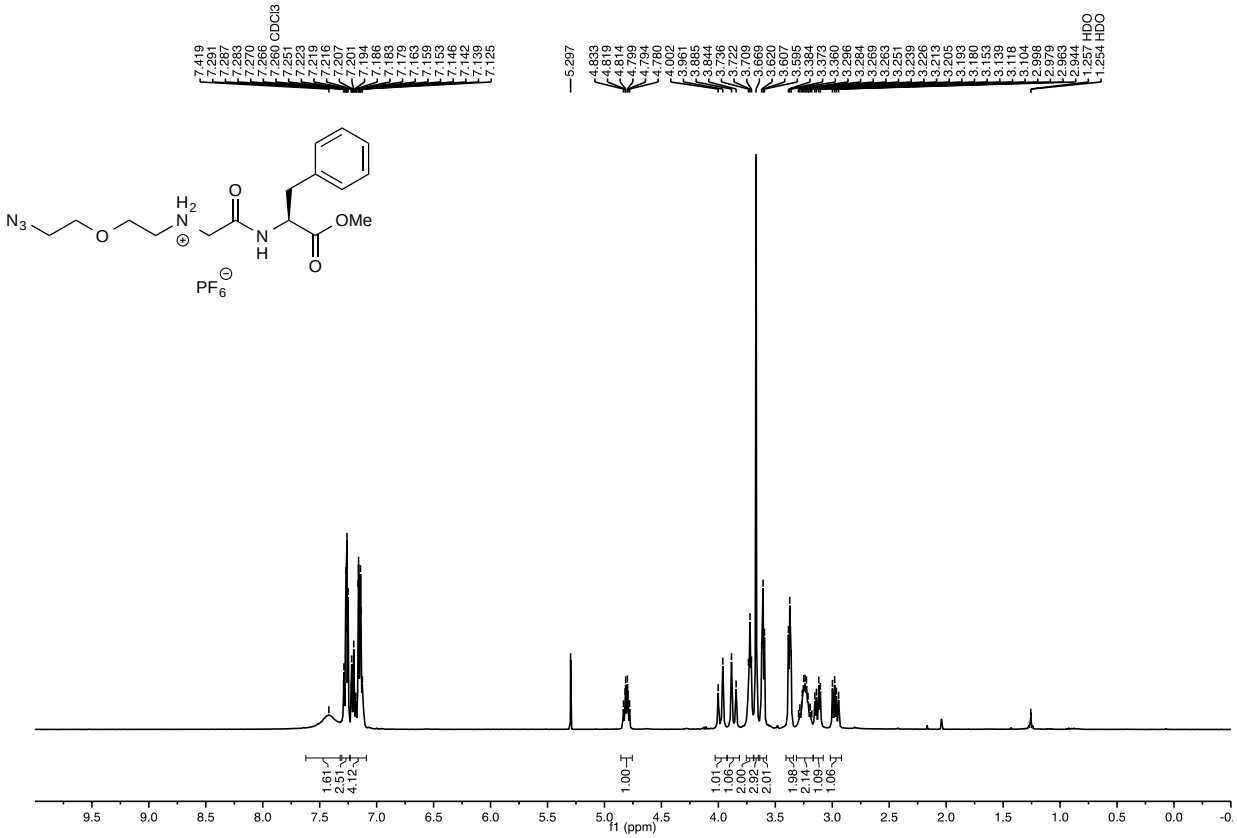


^{13}C NMR (100 MHz, $CDCl_3$)

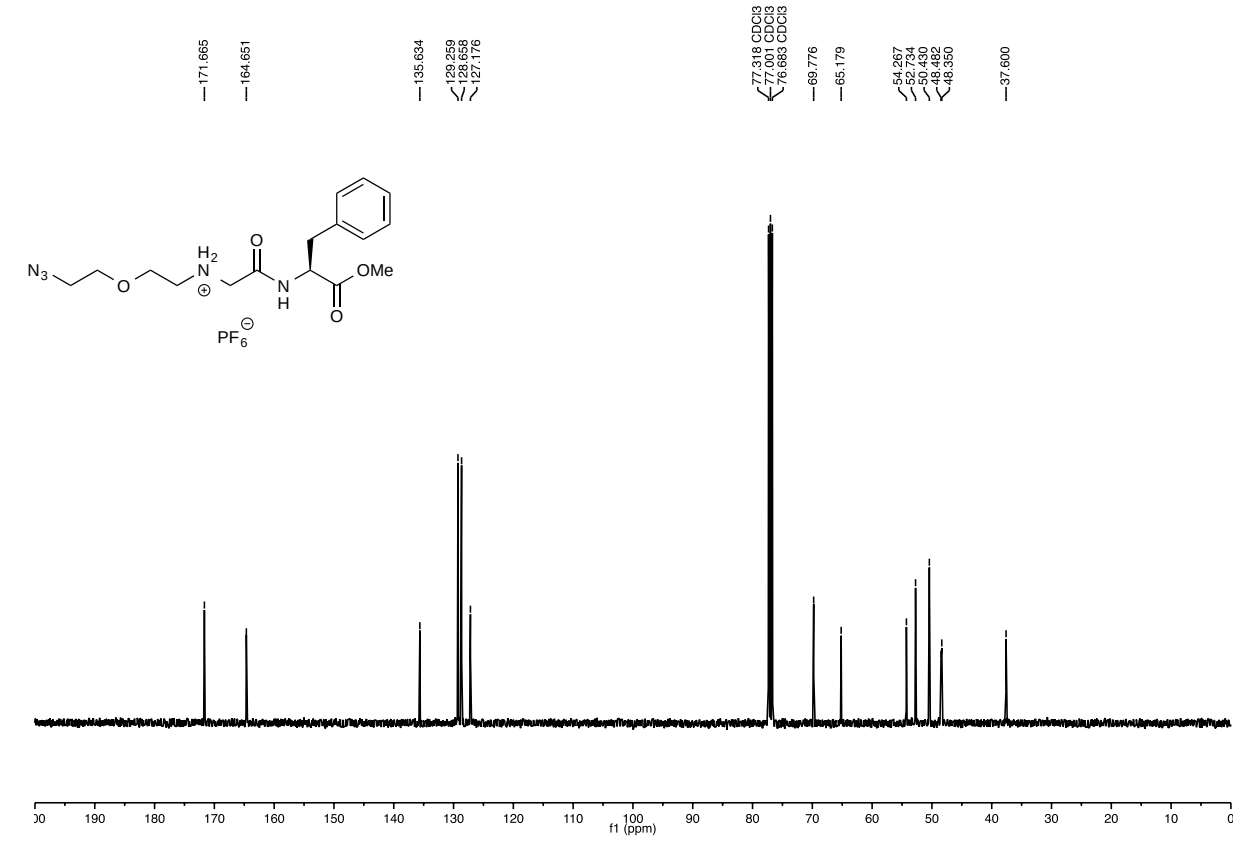


Azide-PF_6 salt S7

^{1}H NMR (400 MHz, $CDCl_3$)

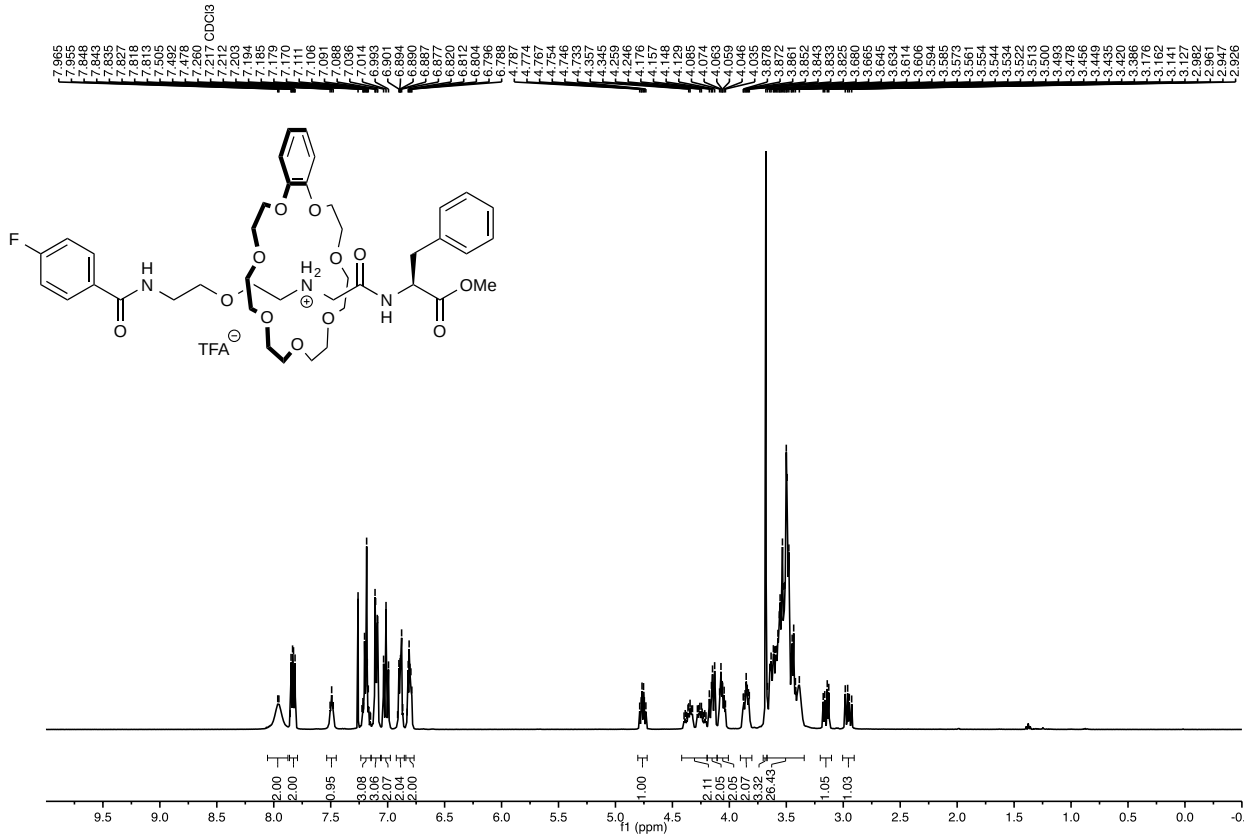


^{13}C NMR (100 MHz, $CDCl_3$)

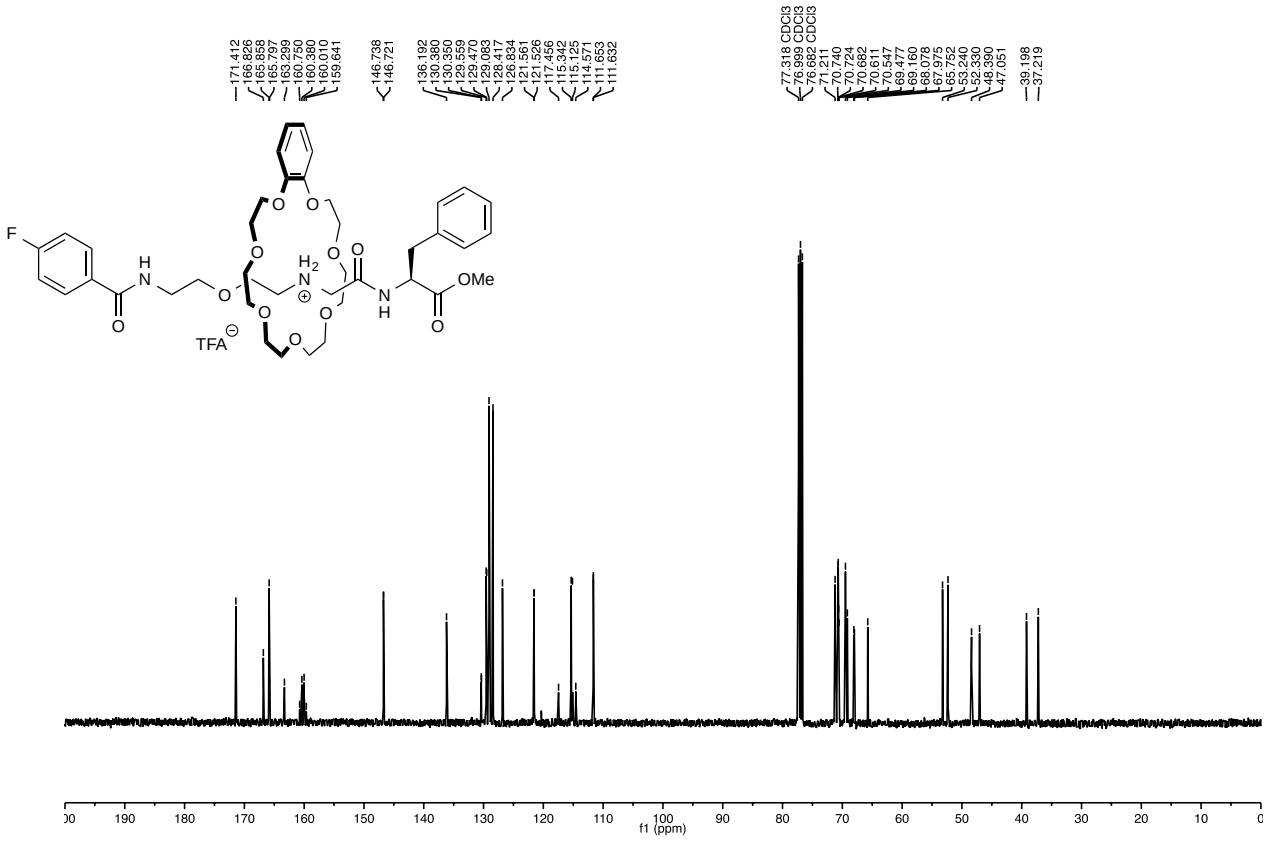


[2]Rotaxane S9-TFA

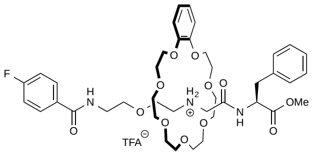
^{1}H NMR (400 MHz, $CDCl_3$)

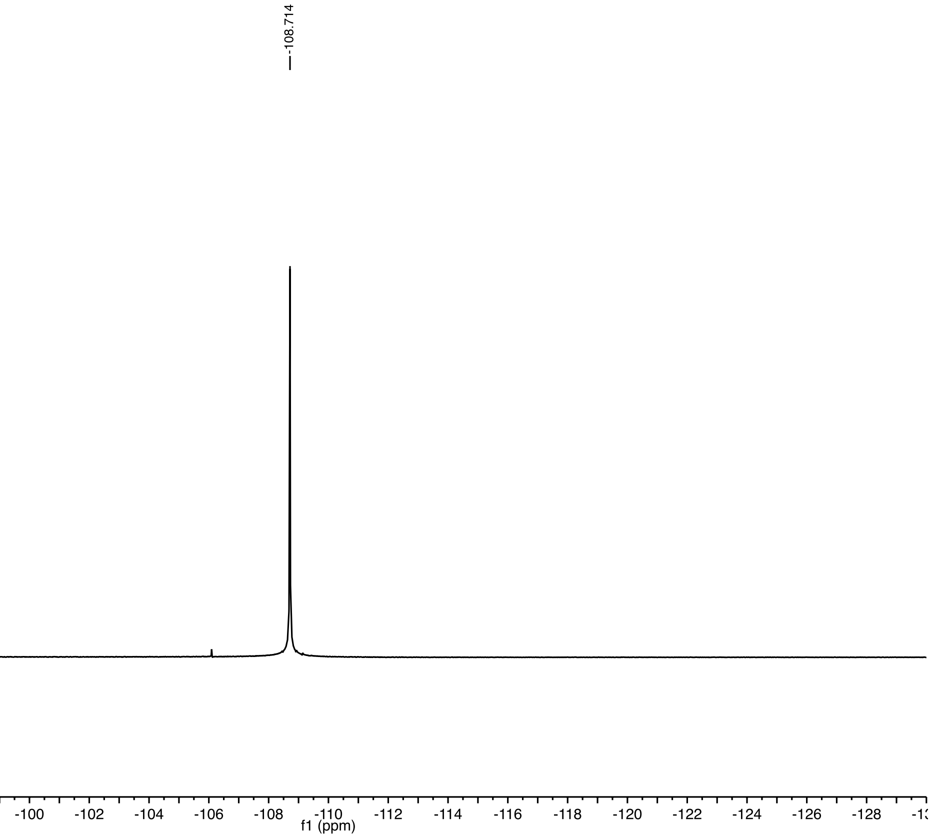


^{13}C NMR (100 MHz, $CDCl_3$)



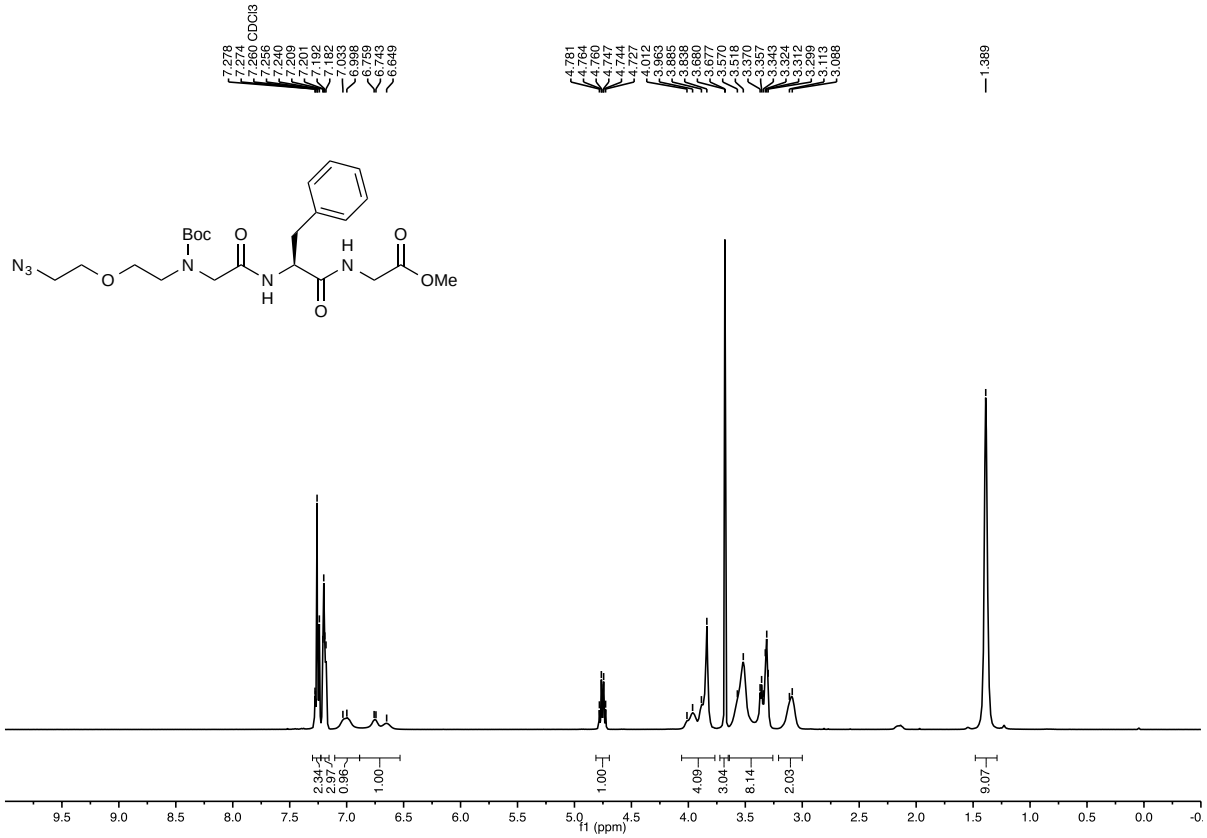
^{19}F NMR (376 MHz, $CDCl_3$)



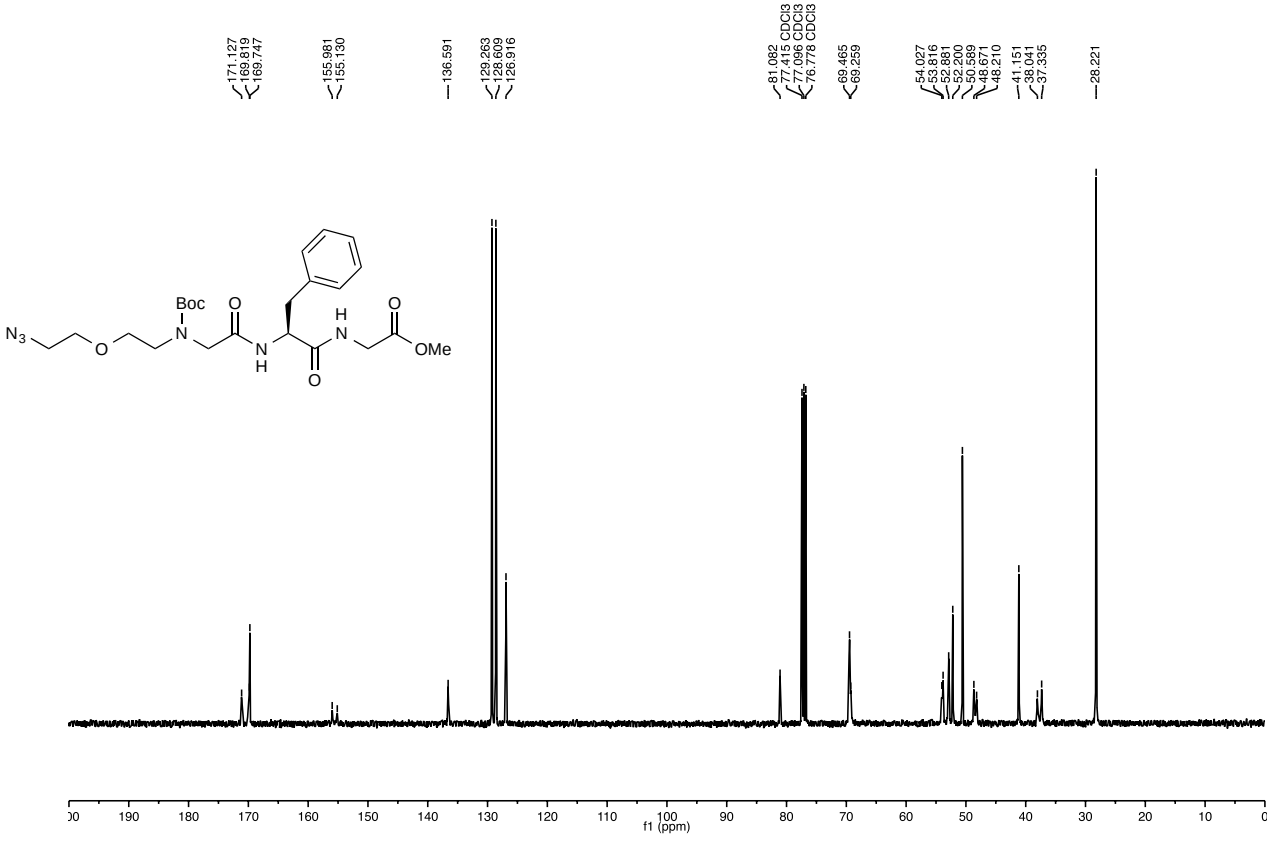


Azide-Phe-Gly methyl ester S10

^{1}H NMR (400 MHz, $CDCl_3$)

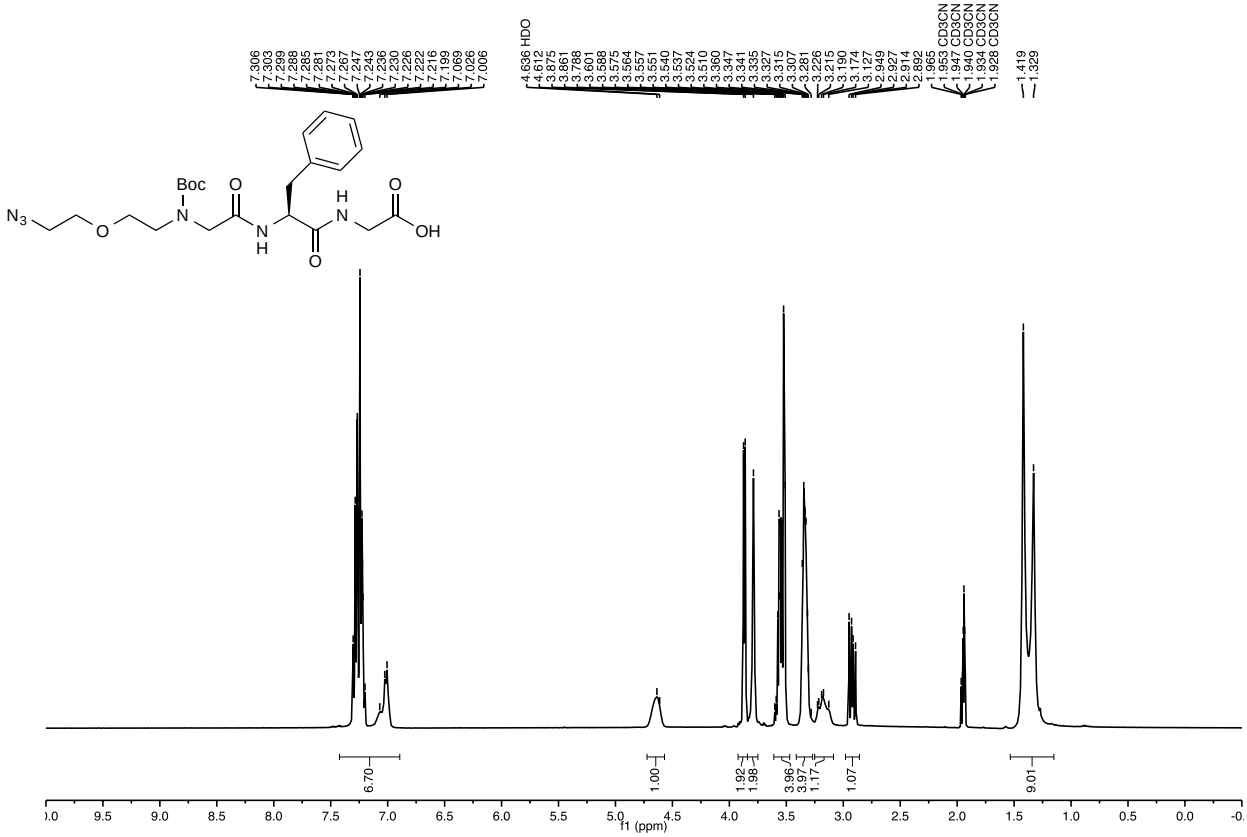


^{13}C NMR (100 MHz, $CDCl_3$)

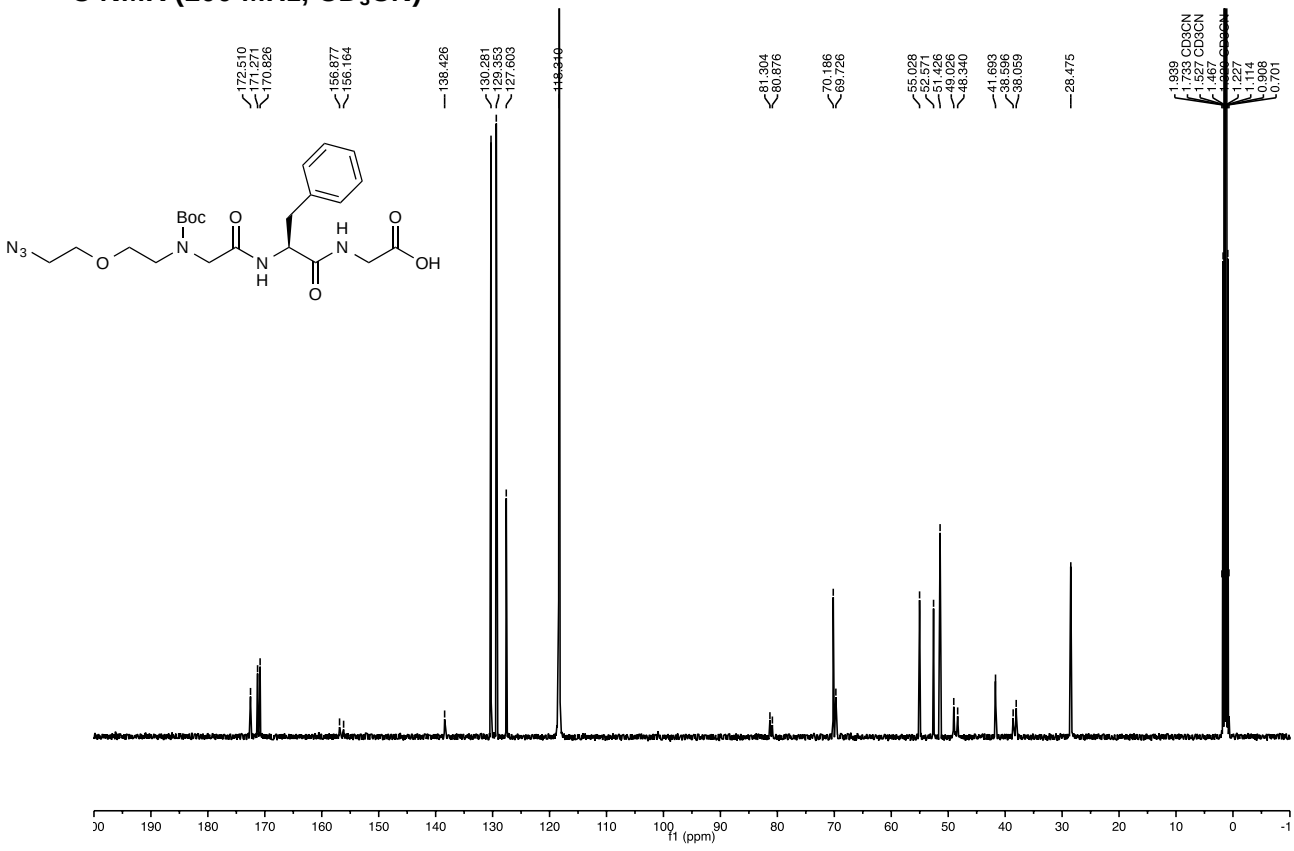


Azide-Phe-Gly acid S11

^{1}H NMR (400 MHz, CD_3CN)

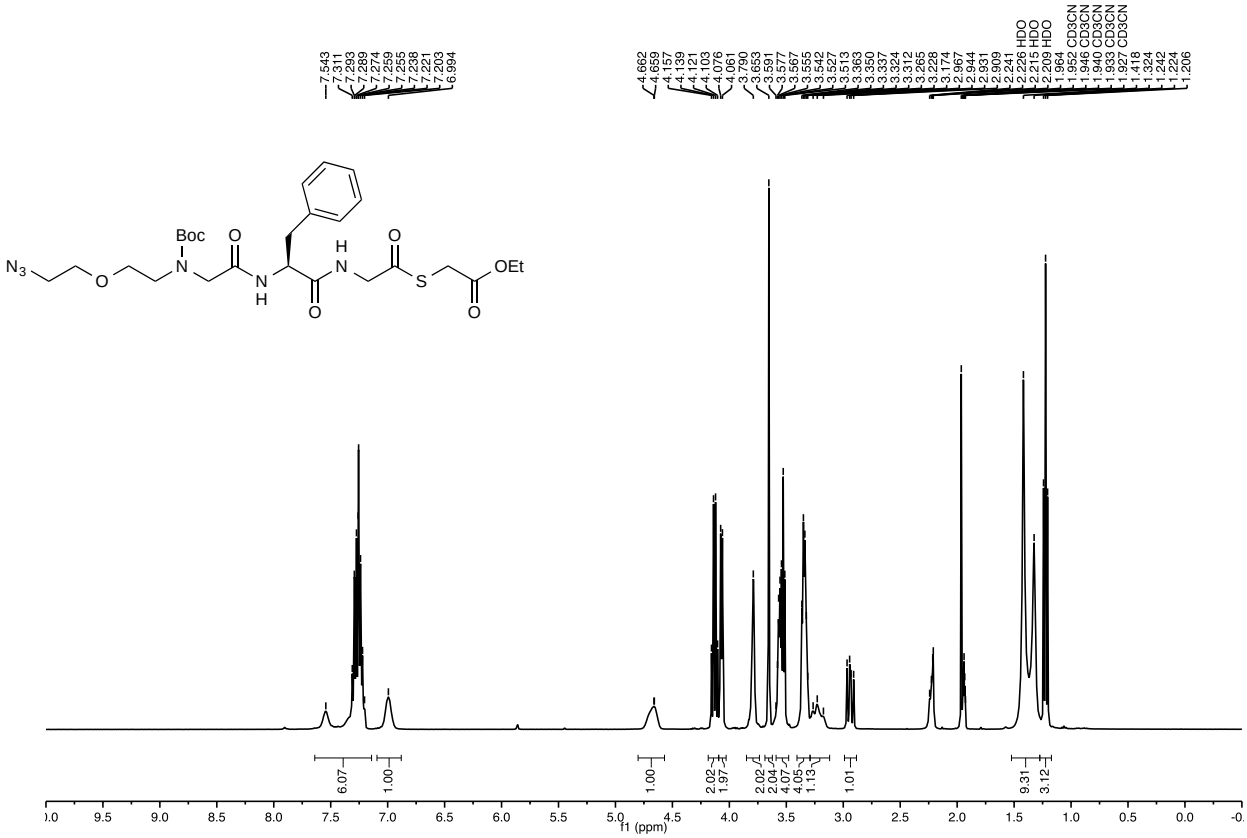


^{13}C NMR (100 MHz, CD_3CN)

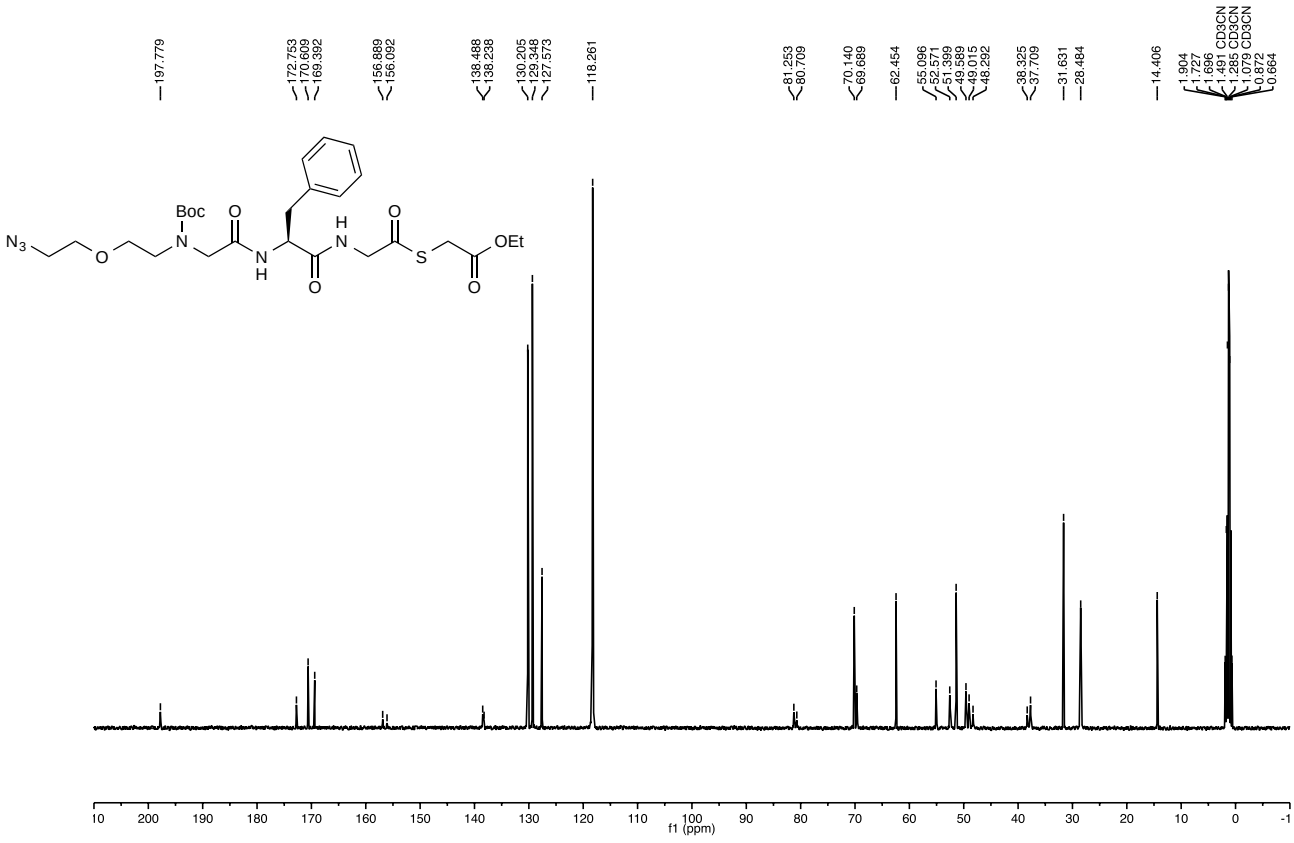


Azide-Phe-Gly thioester S12

^{1}H NMR (400 MHz, CD_3CN)

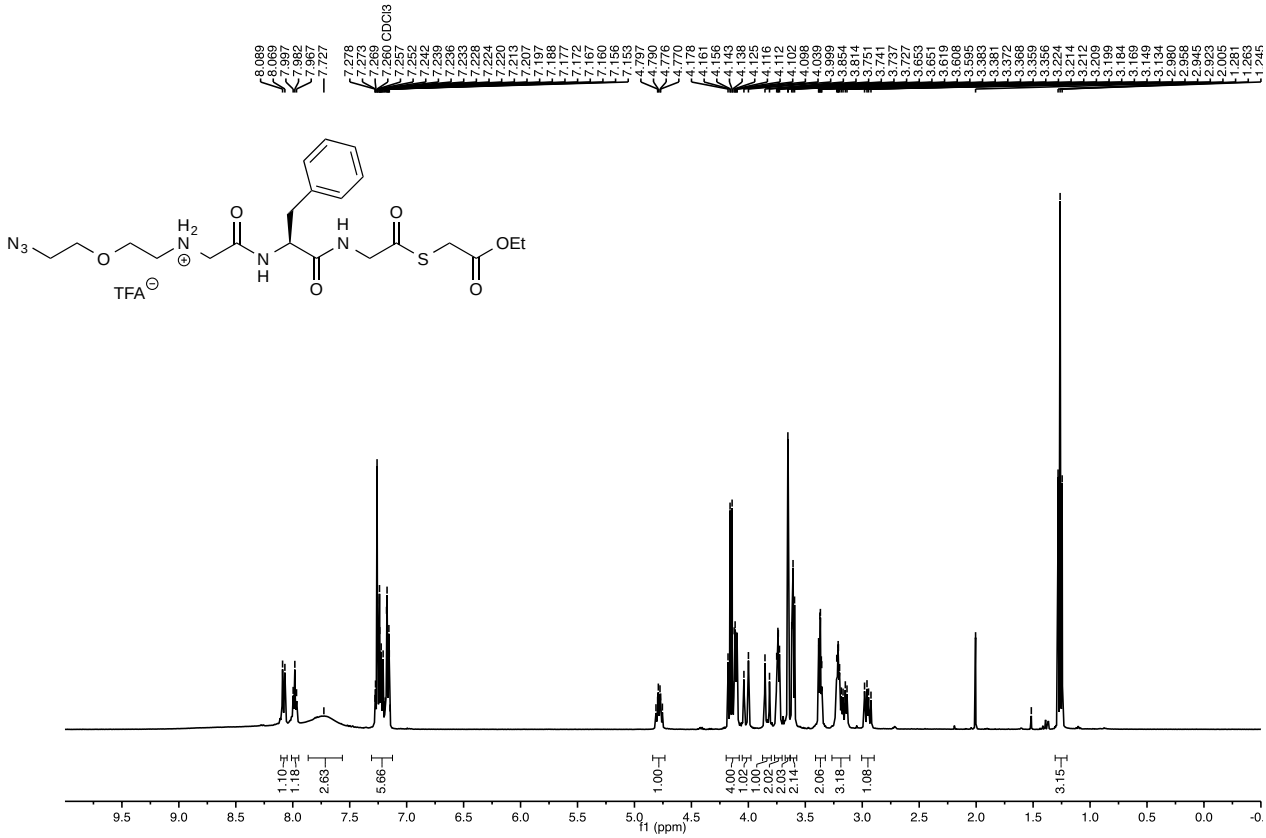


^{13}C NMR (100 MHz, CD_3CN)

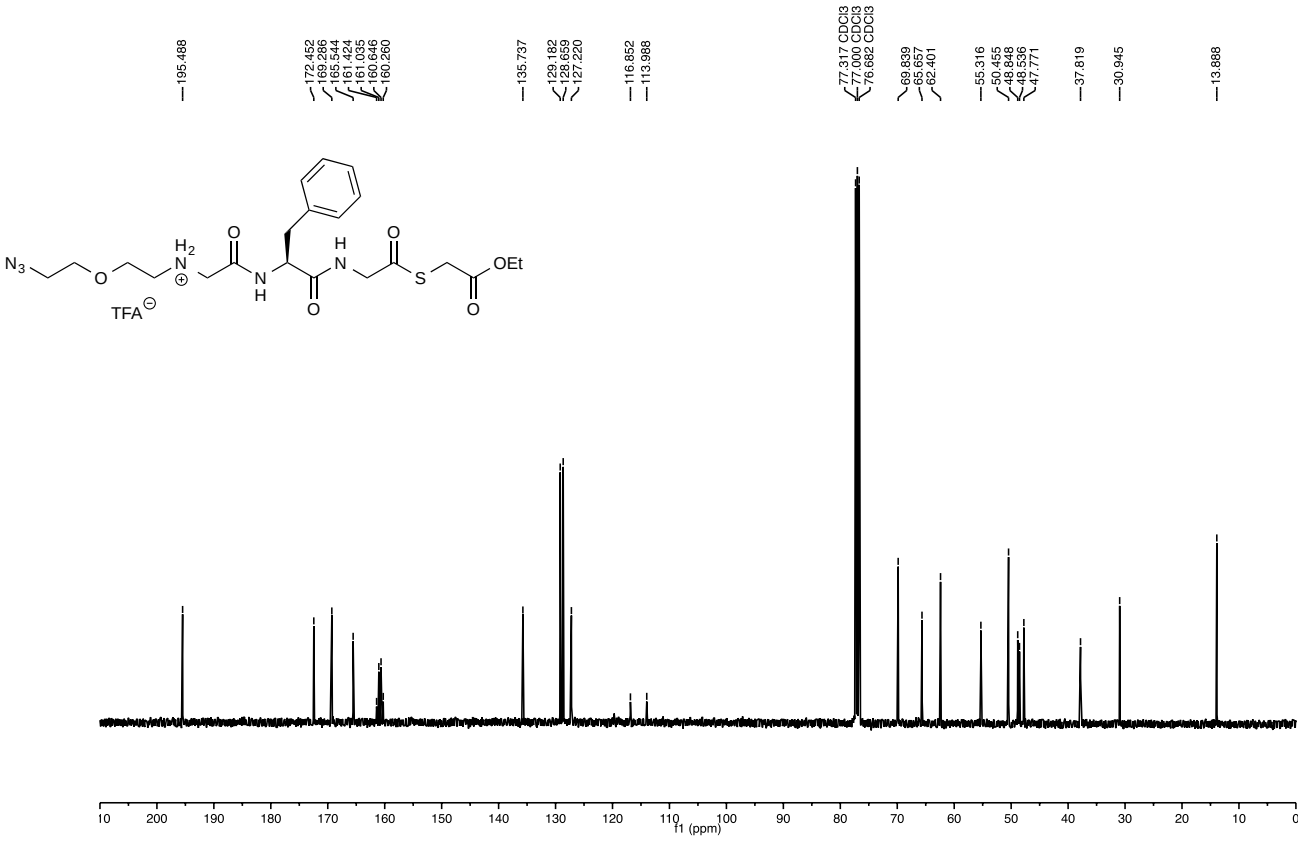


Azide-Phe-Gly thioester TFA salt 3

^{1}H NMR (400 MHz, $CDCl_3$)

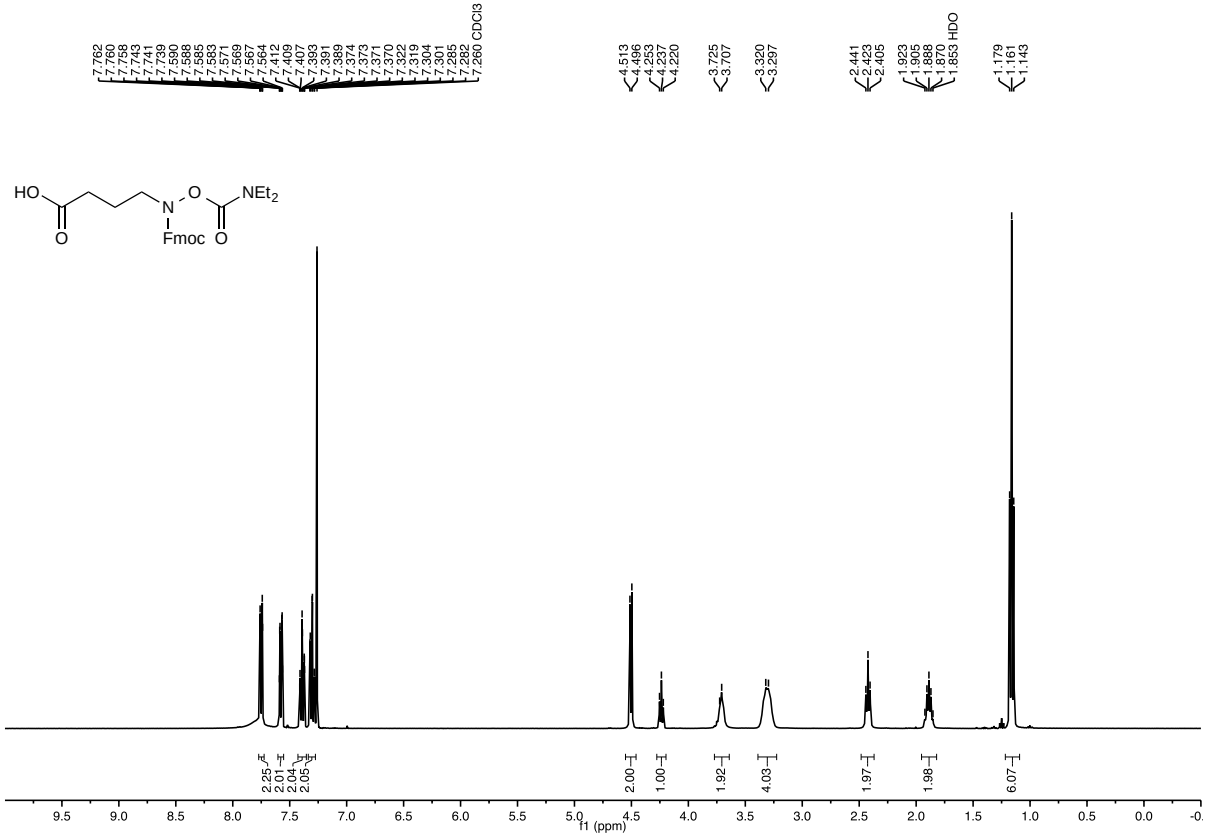


^{13}C NMR (100 MHz, $CDCl_3$)

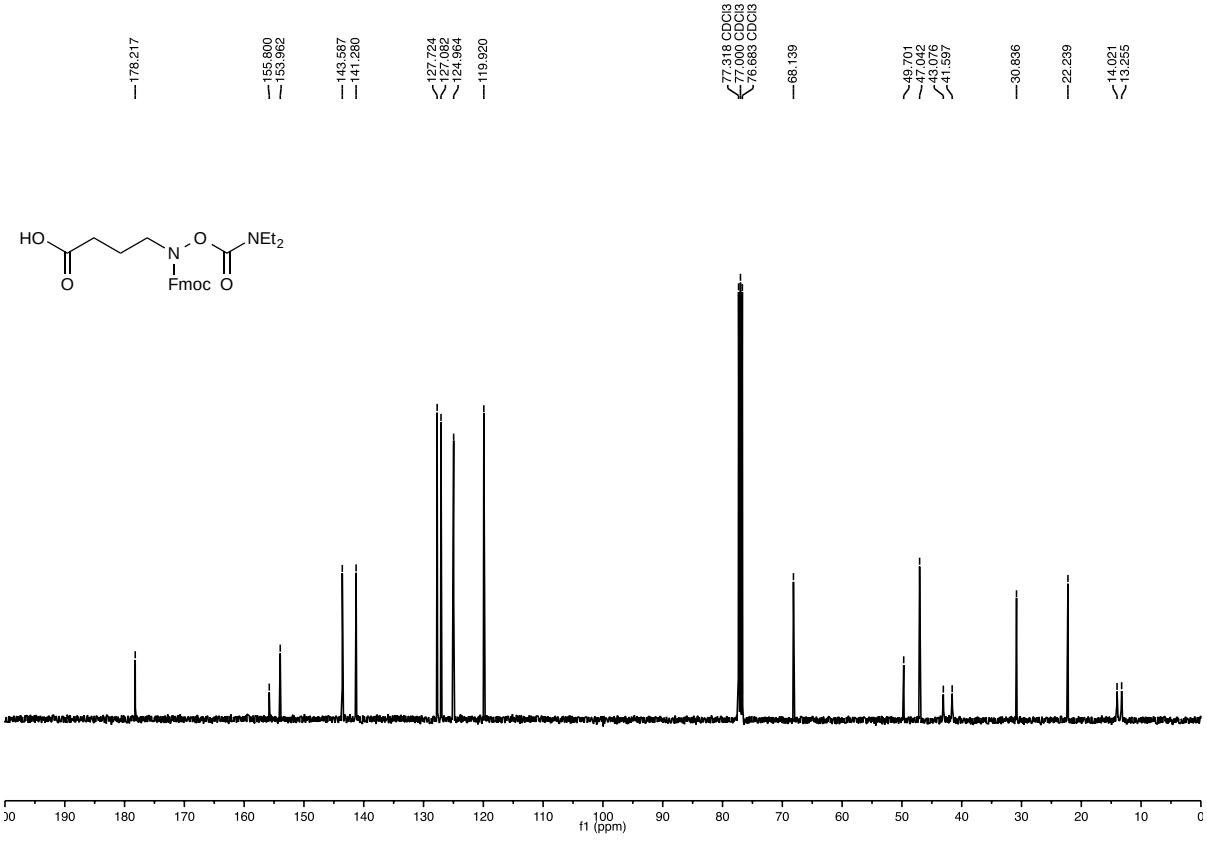


N-Fmoc hydroxylamine S14

^{1}H NMR (400 MHz, $CDCl_3$)

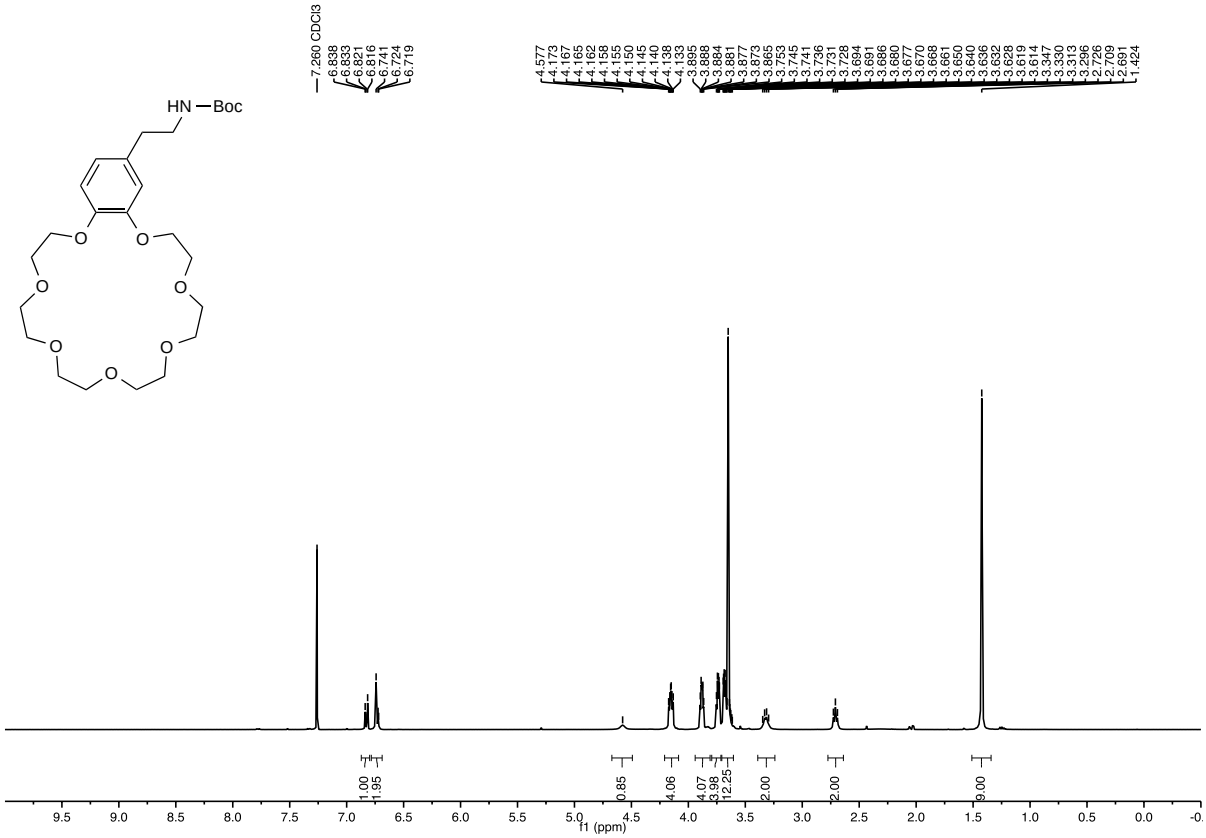


^{13}C NMR (100 MHz, $CDCl_3$)

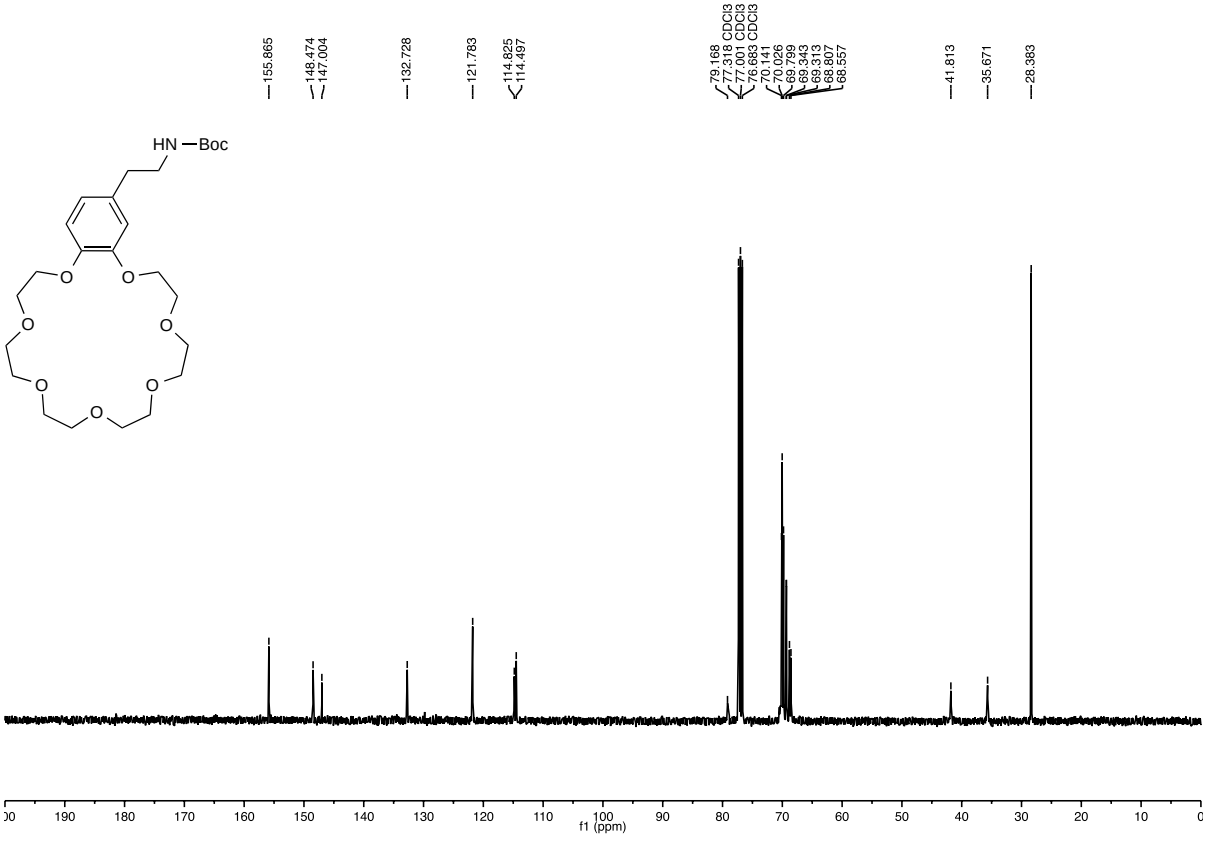


B21C7-*N*-Boc amine S17

^{1}H NMR (400 MHz, $CDCl_3$)

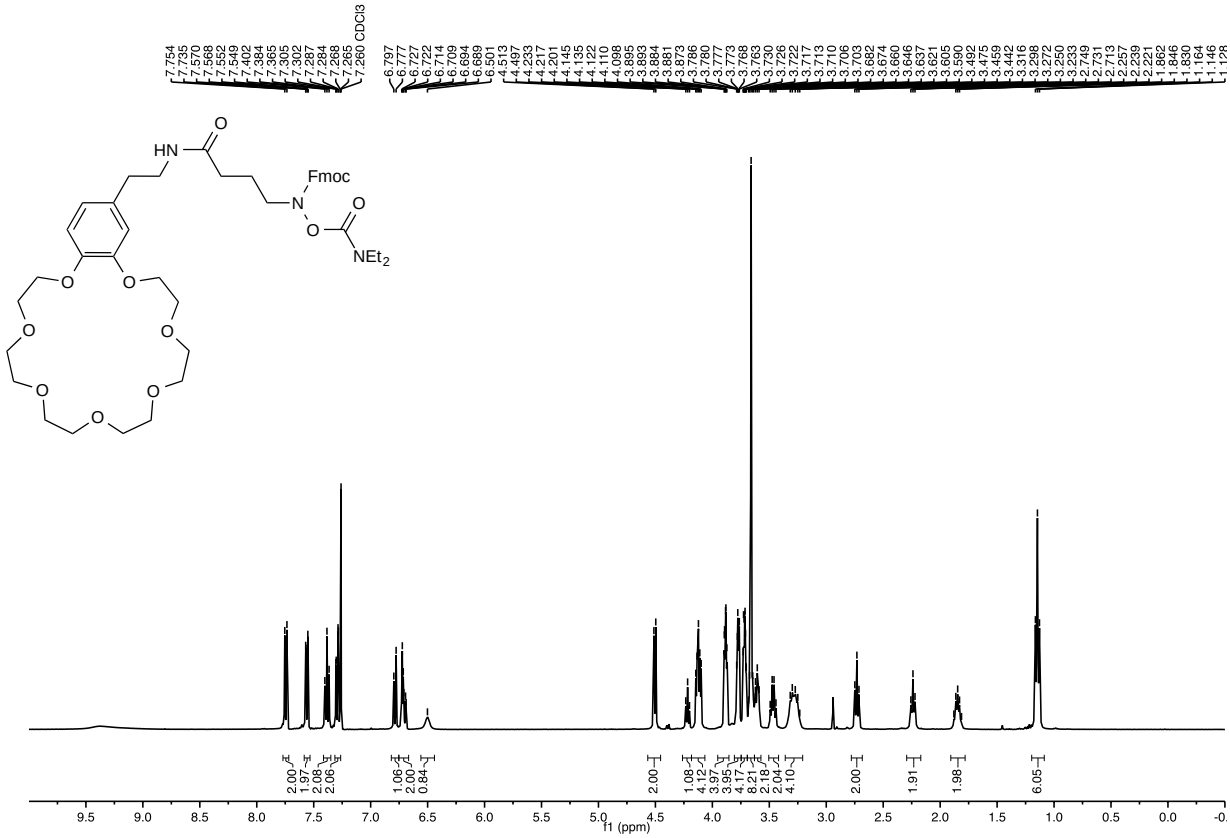


^{13}C NMR (100 MHz, $CDCl_3$)

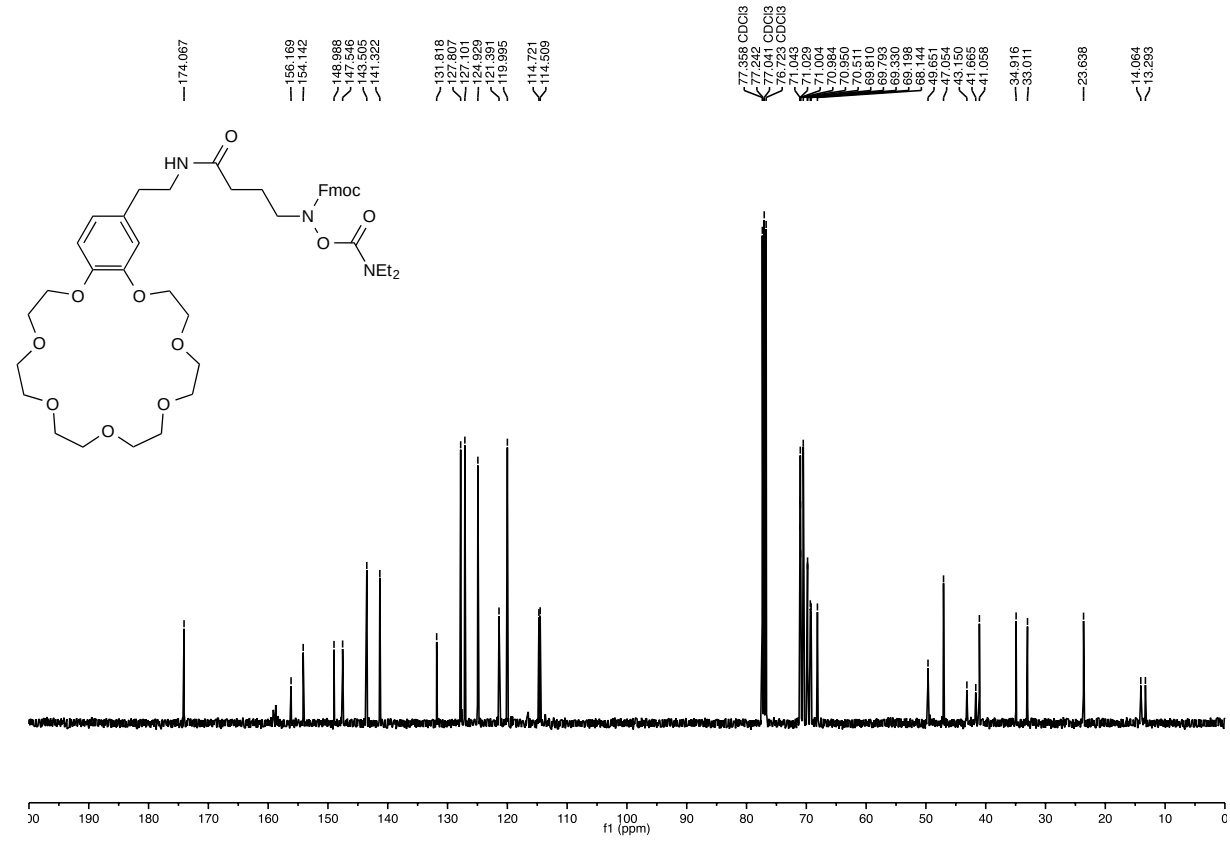


B21C7-*N*-Fmoc hydroxylamine 2

^{1}H NMR (400 MHz, $CDCl_3$)

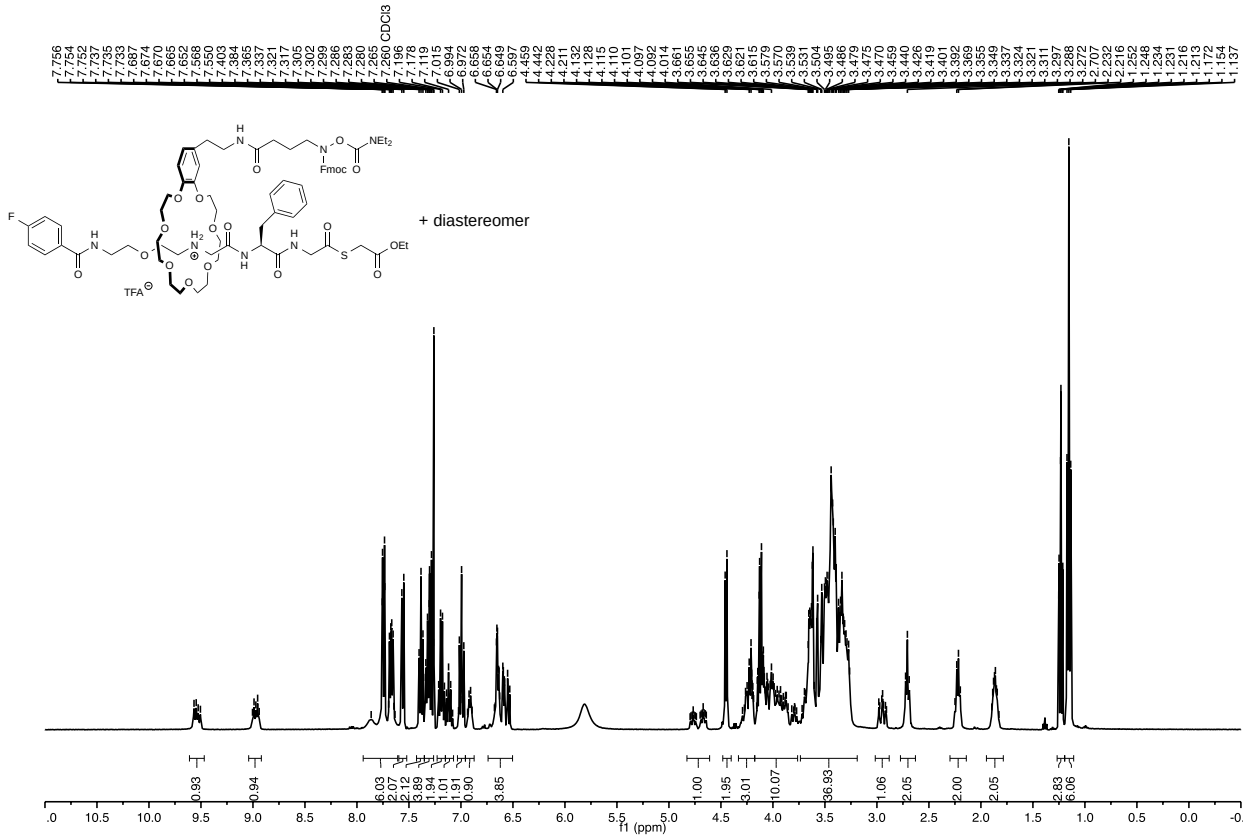


^{13}C NMR (100 MHz, $CDCl_3$)

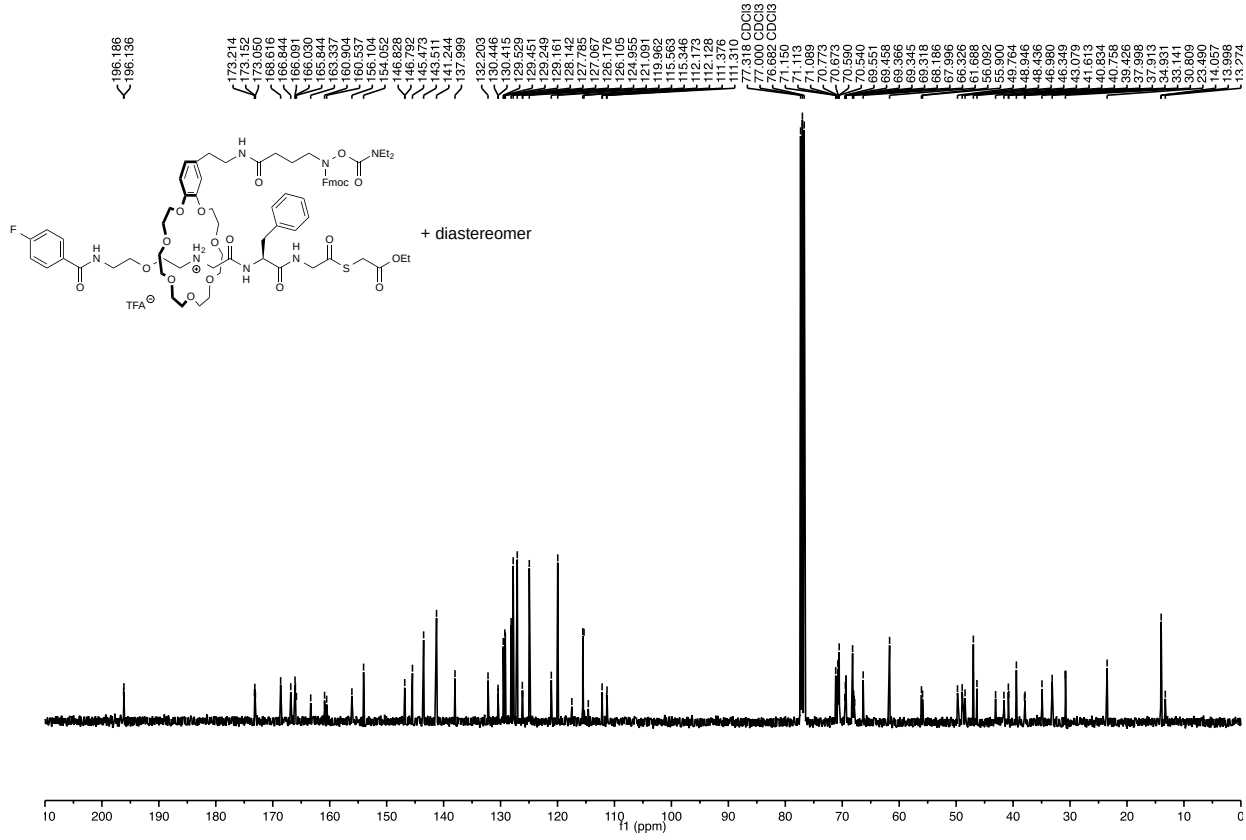


[2]Rotaxane 4

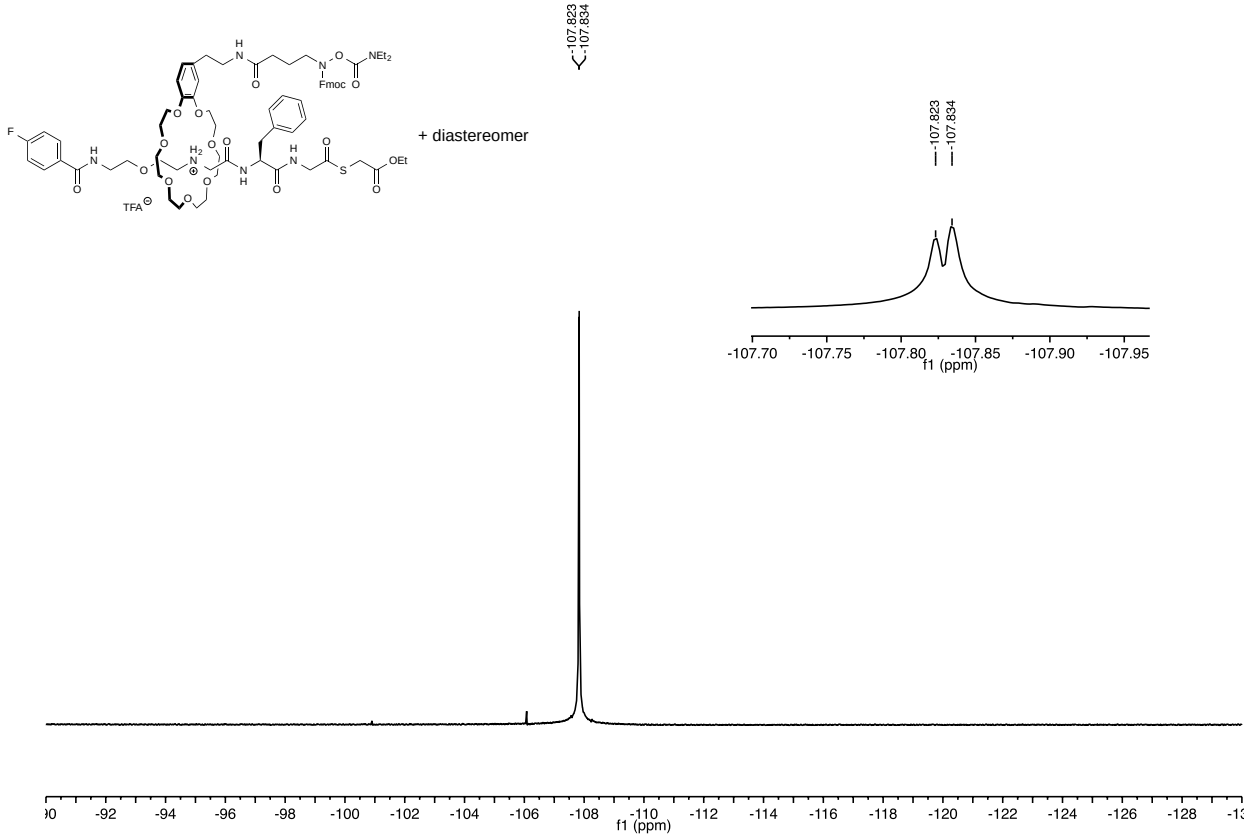
^{1}H NMR (400 MHz, $CDCl_3$)



^{13}C NMR (100 MHz, $CDCl_3$)

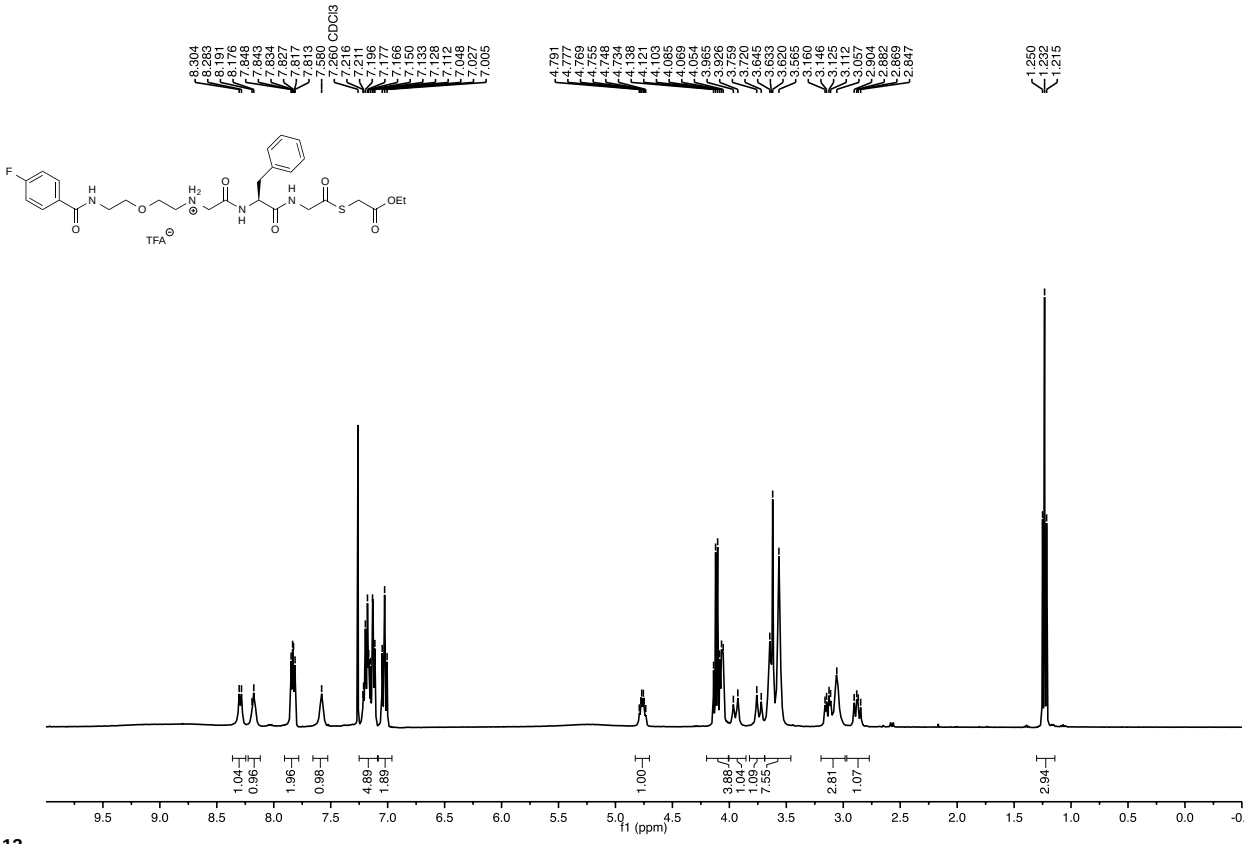


^{19}F NMR (376 MHz, $CDCl_3$)

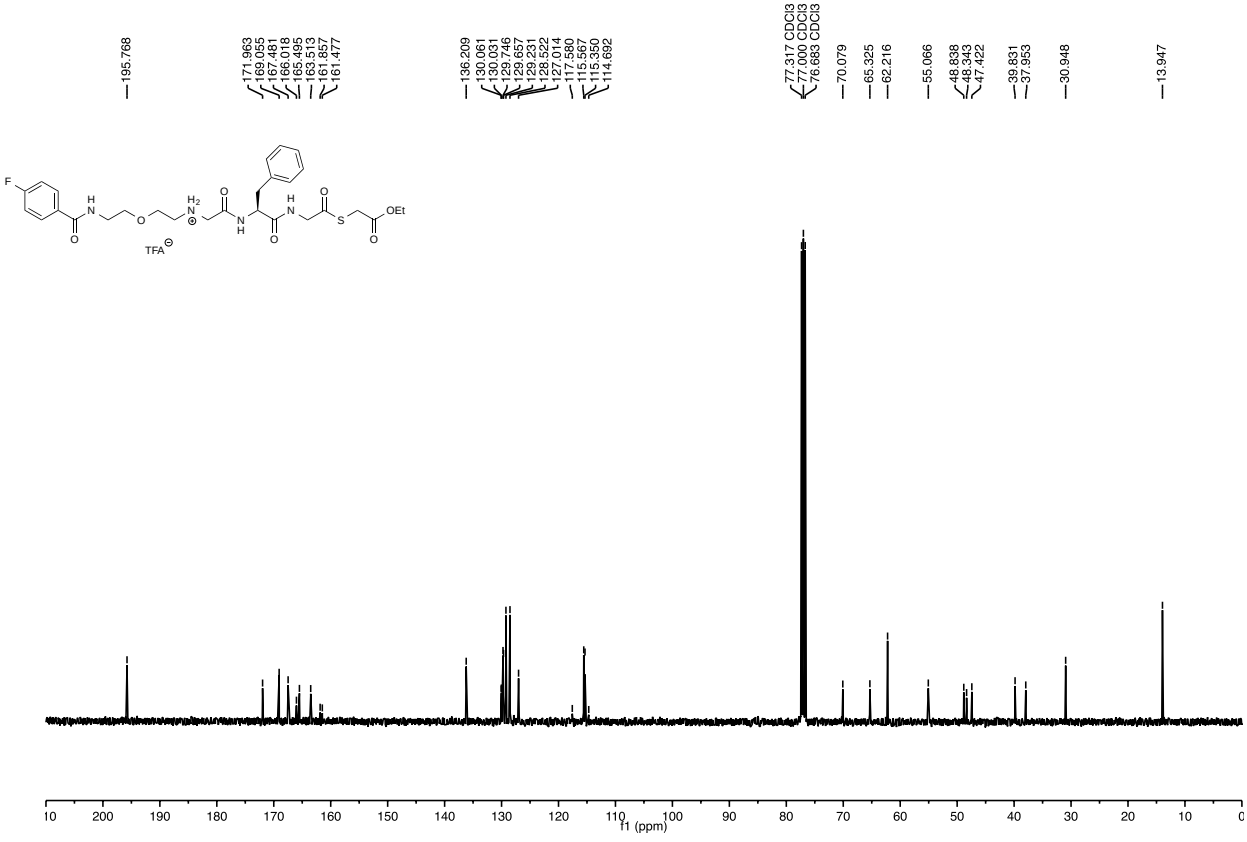


Axle 5

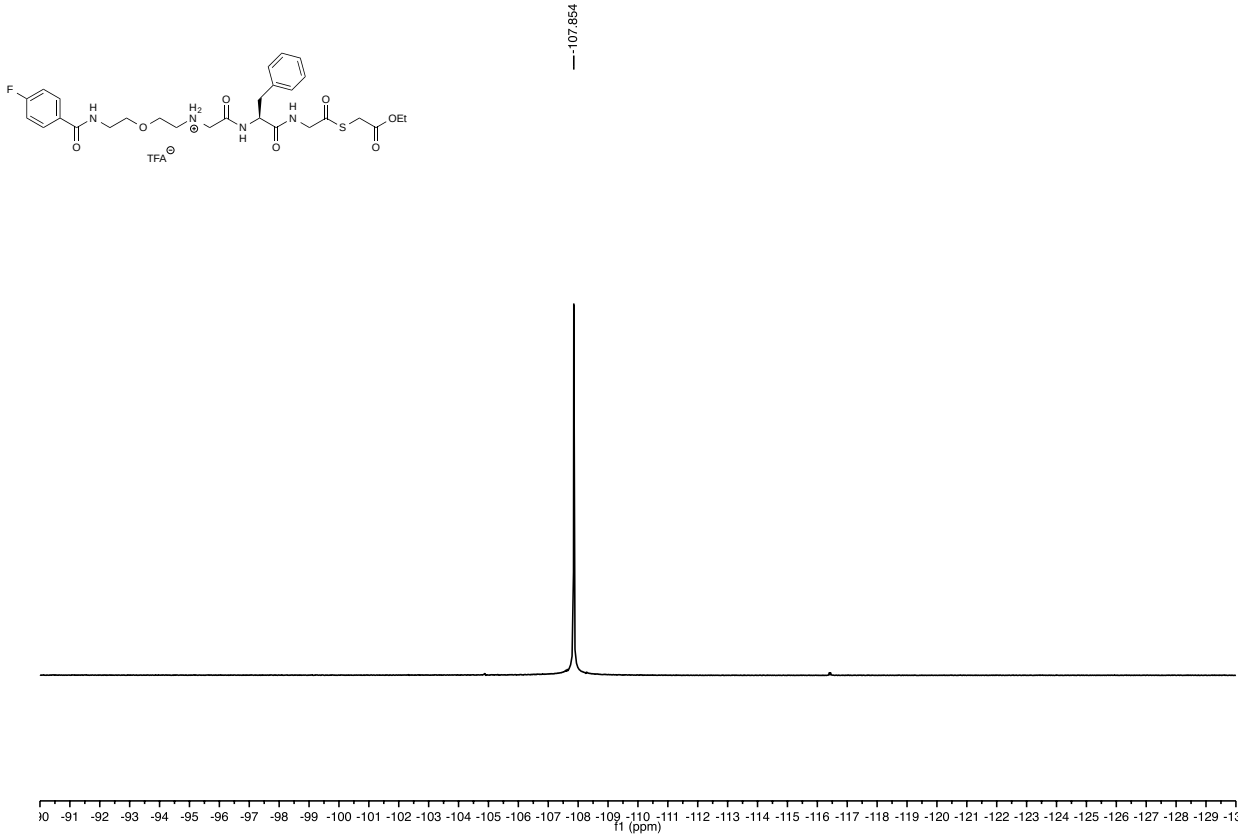
^{1}H NMR (400 MHz, $CDCl_3$)



^{13}C NMR (100 MHz, $CDCl_3$)

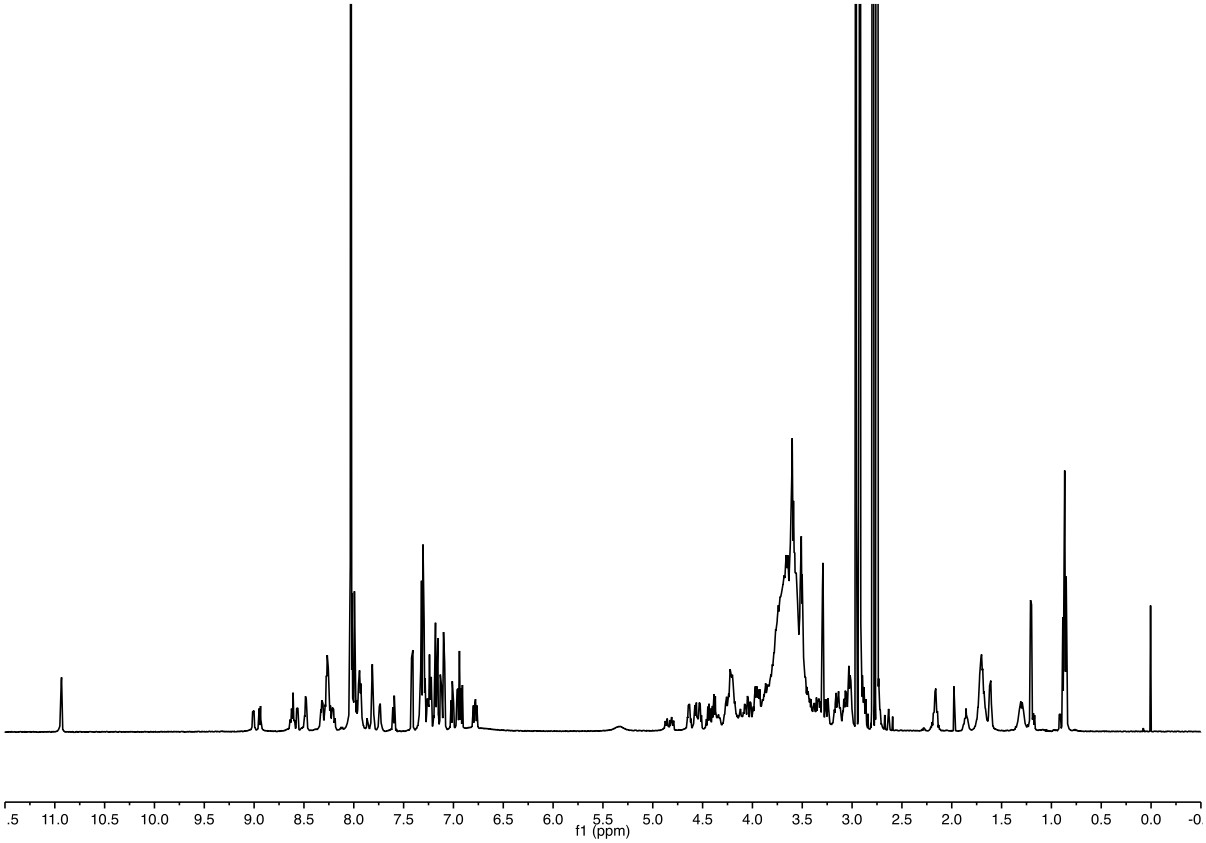


^{19}F NMR (376 MHz, $CDCl_3$)

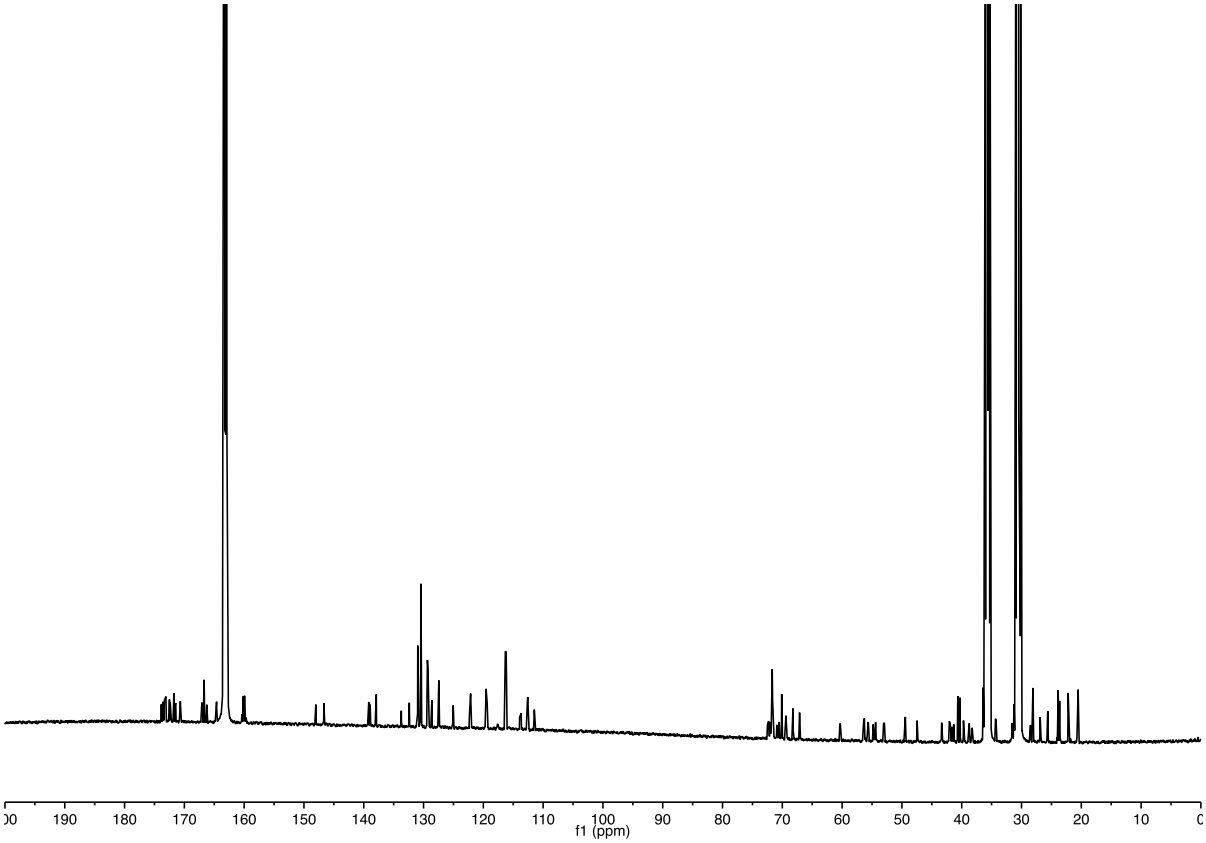


Lasso peptide L1

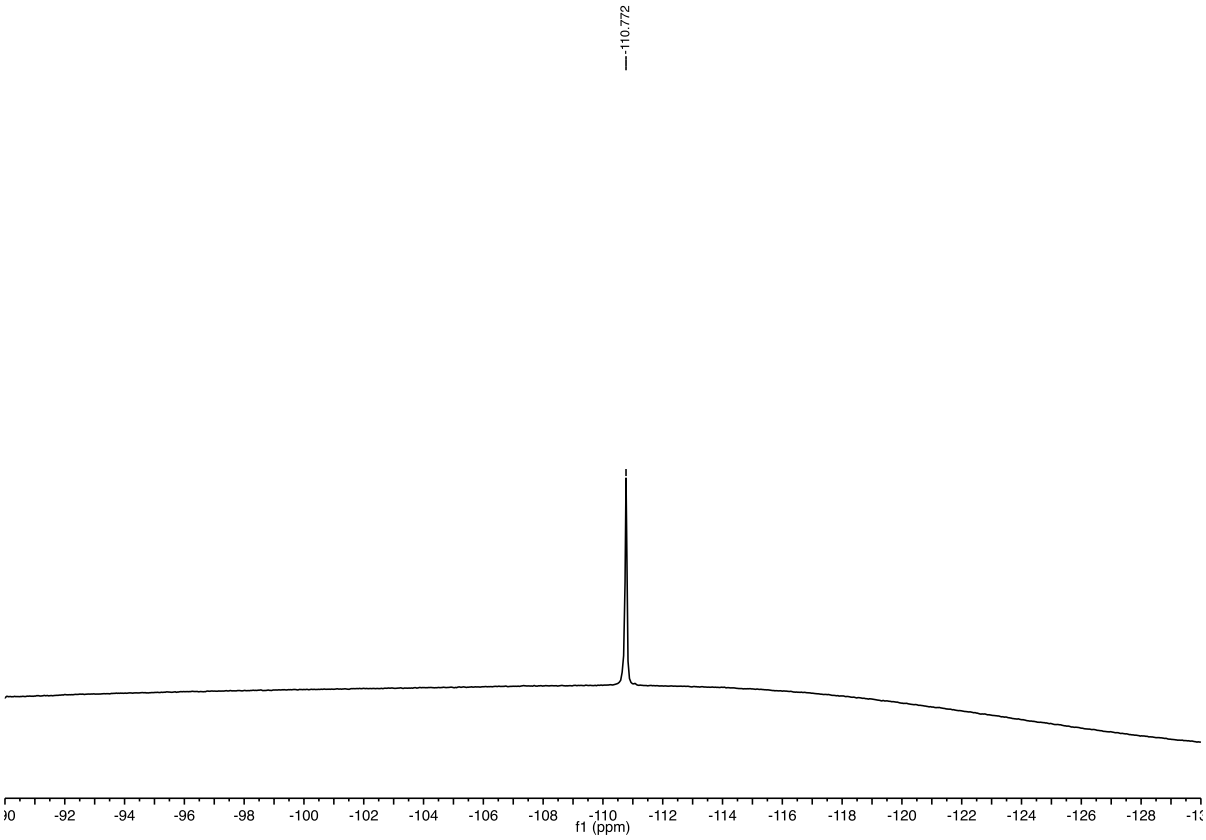
^{1}H NMR (600 MHz, d_7-DMF)



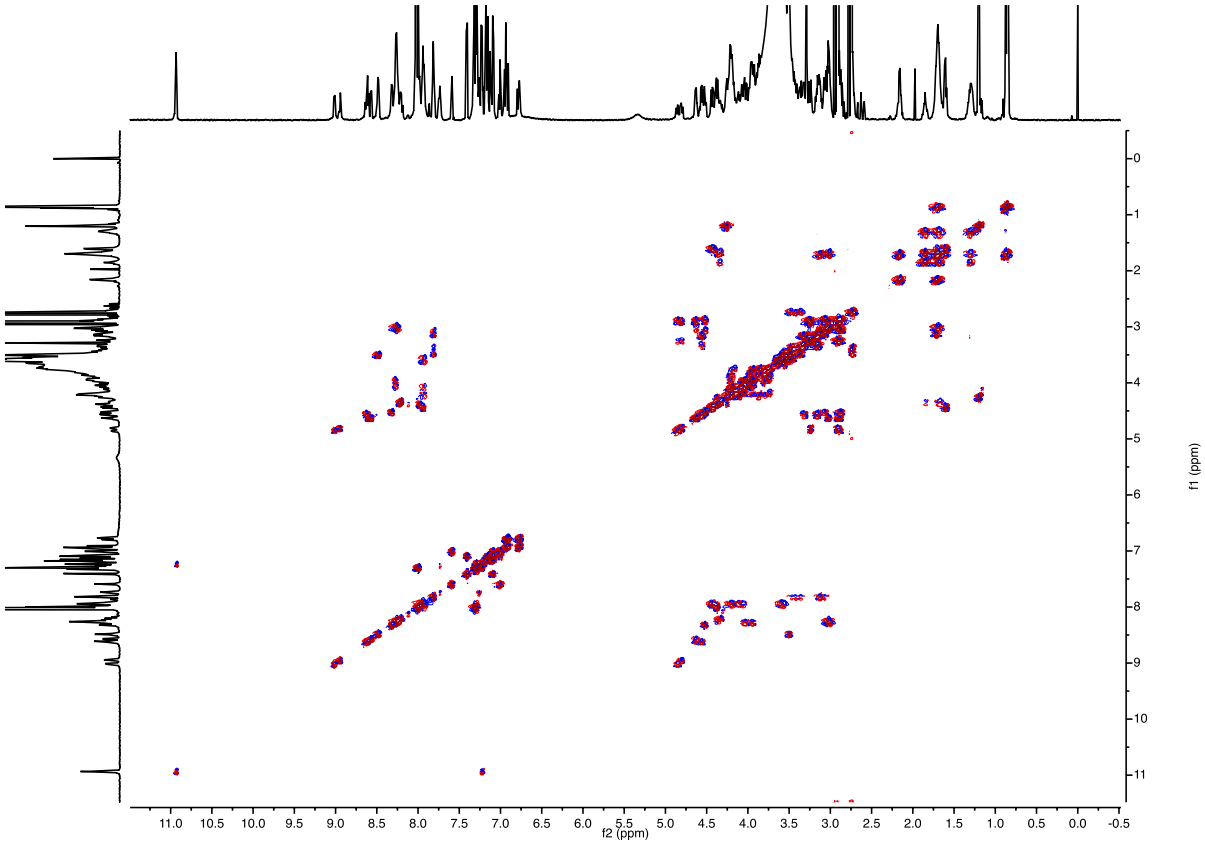
^{13}C NMR (150 MHz, d_7-DMF)



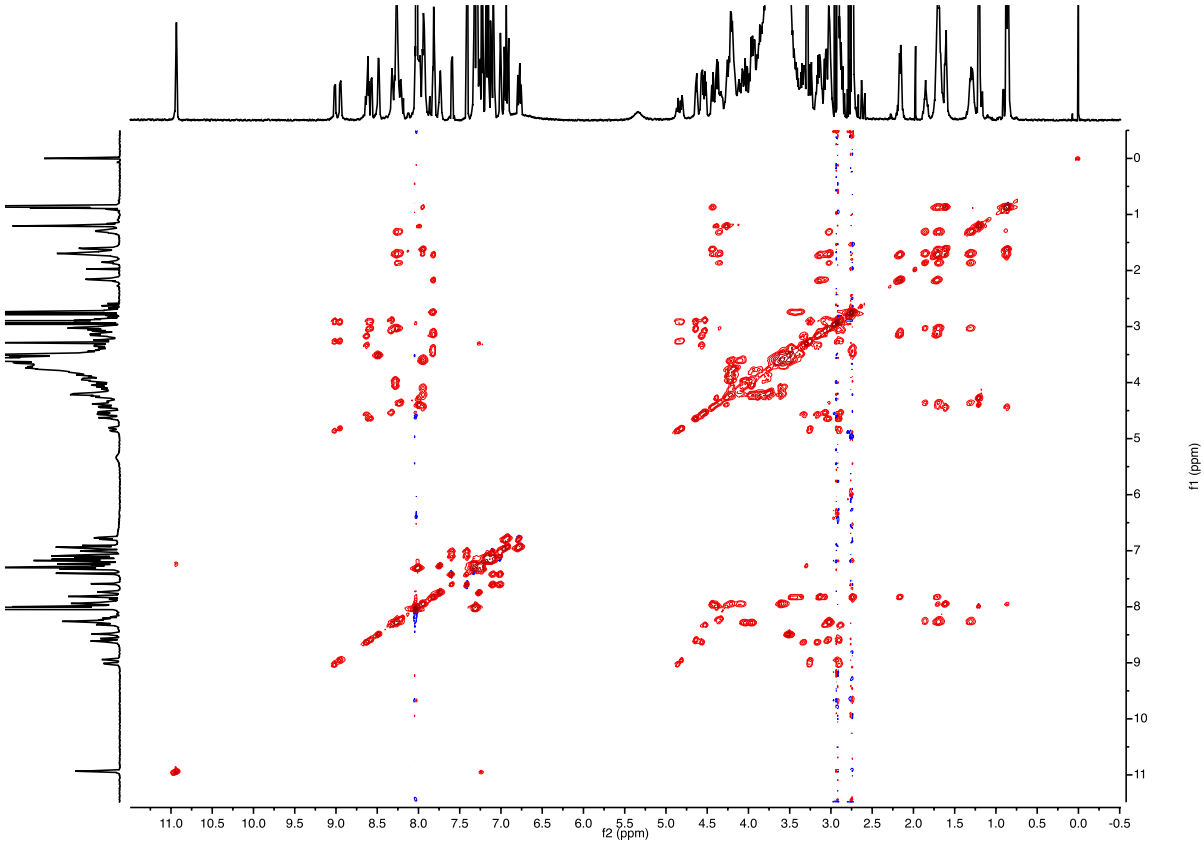
^{19}F NMR (565 MHz, d_7-DMF)



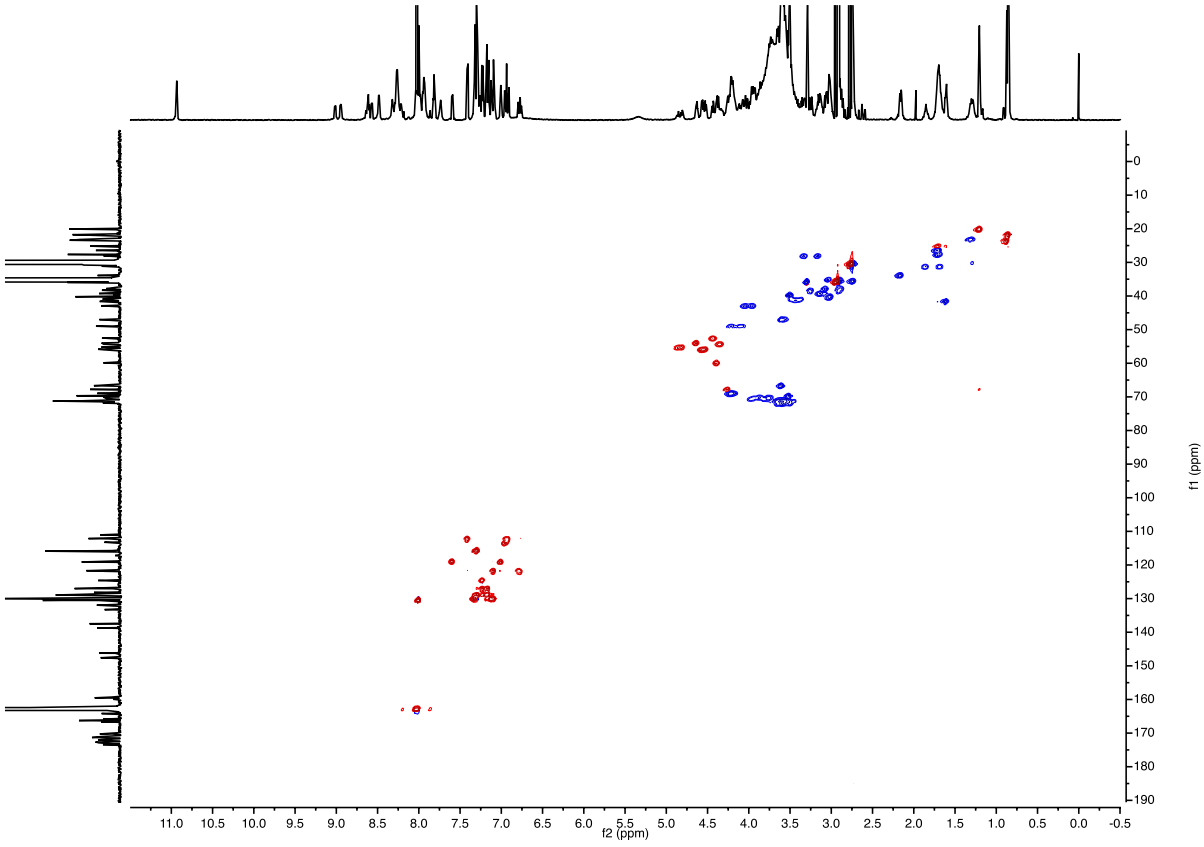
^{1}H-^{1}H DQF-COSY (600 MHz, d_7-DMF)



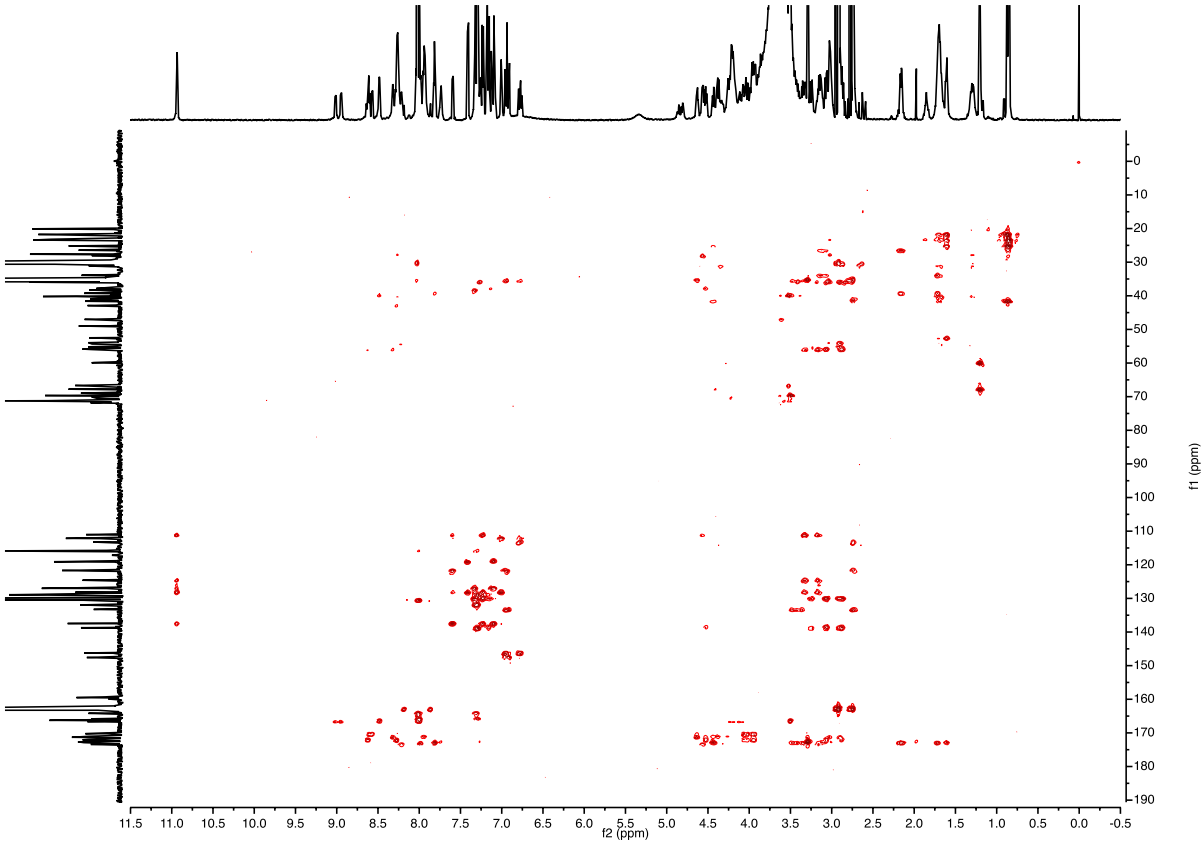
^{1}H-^{1}H TOCSY (600 MHz, d_7-DMF)



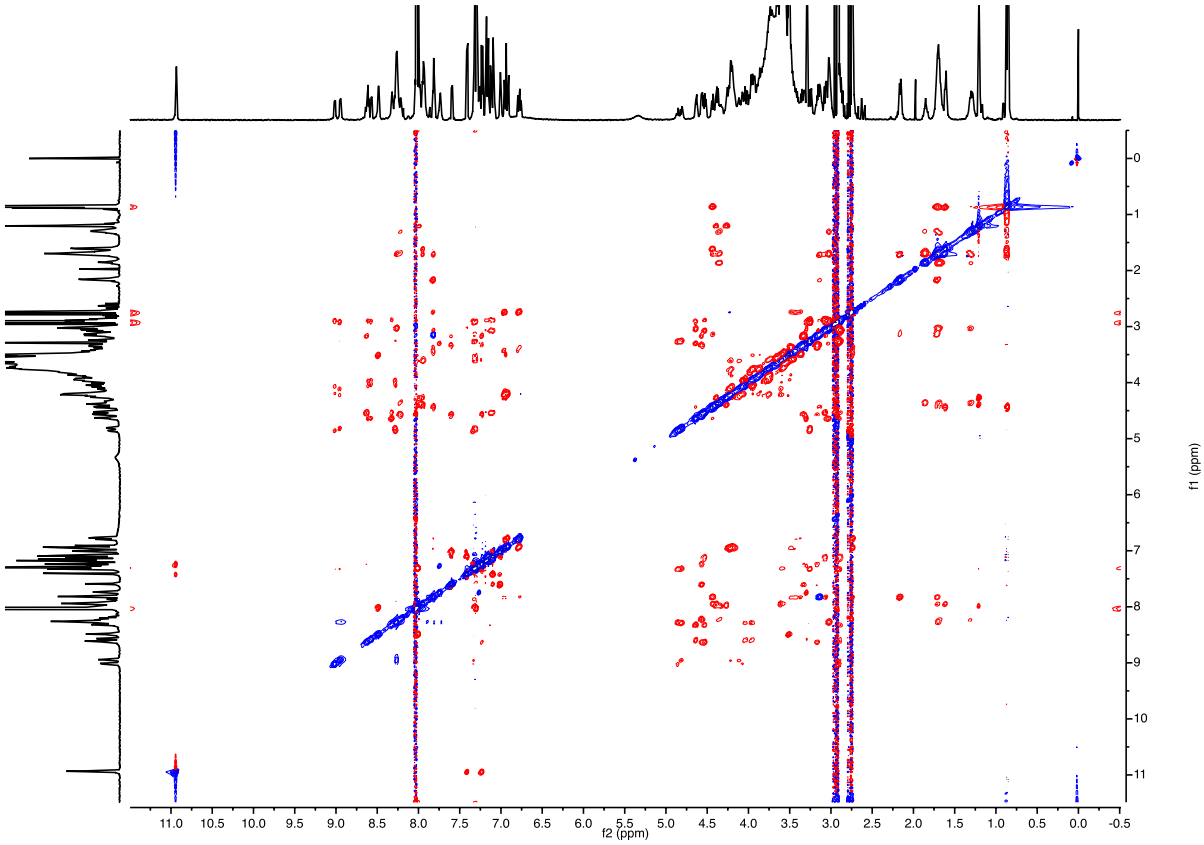
^{1}H-^{13}C HSQC (600 MHz, d_7-DMF)



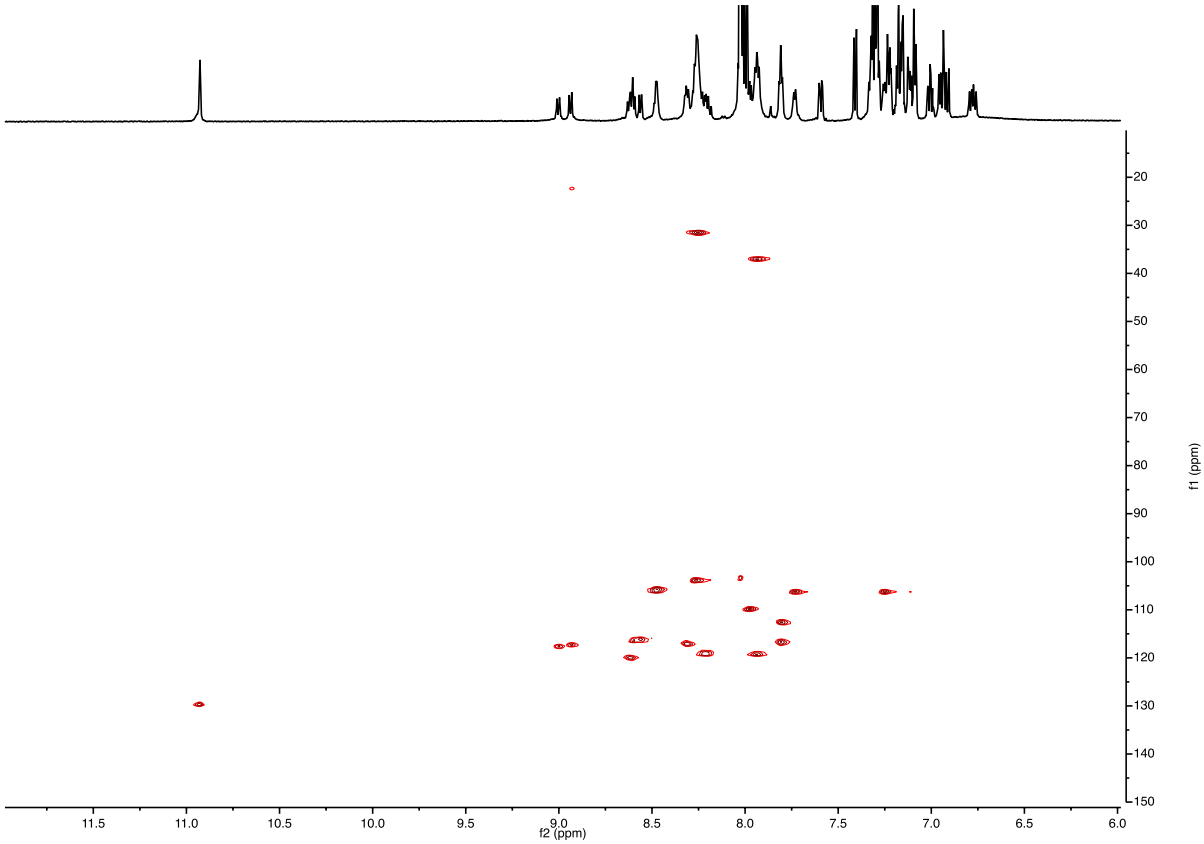
^{1}H-^{13}C HMBC (600 MHz, d_7-DMF)



^{1}H-^{1}H ROESY (600 MHz, d_7-DMF)

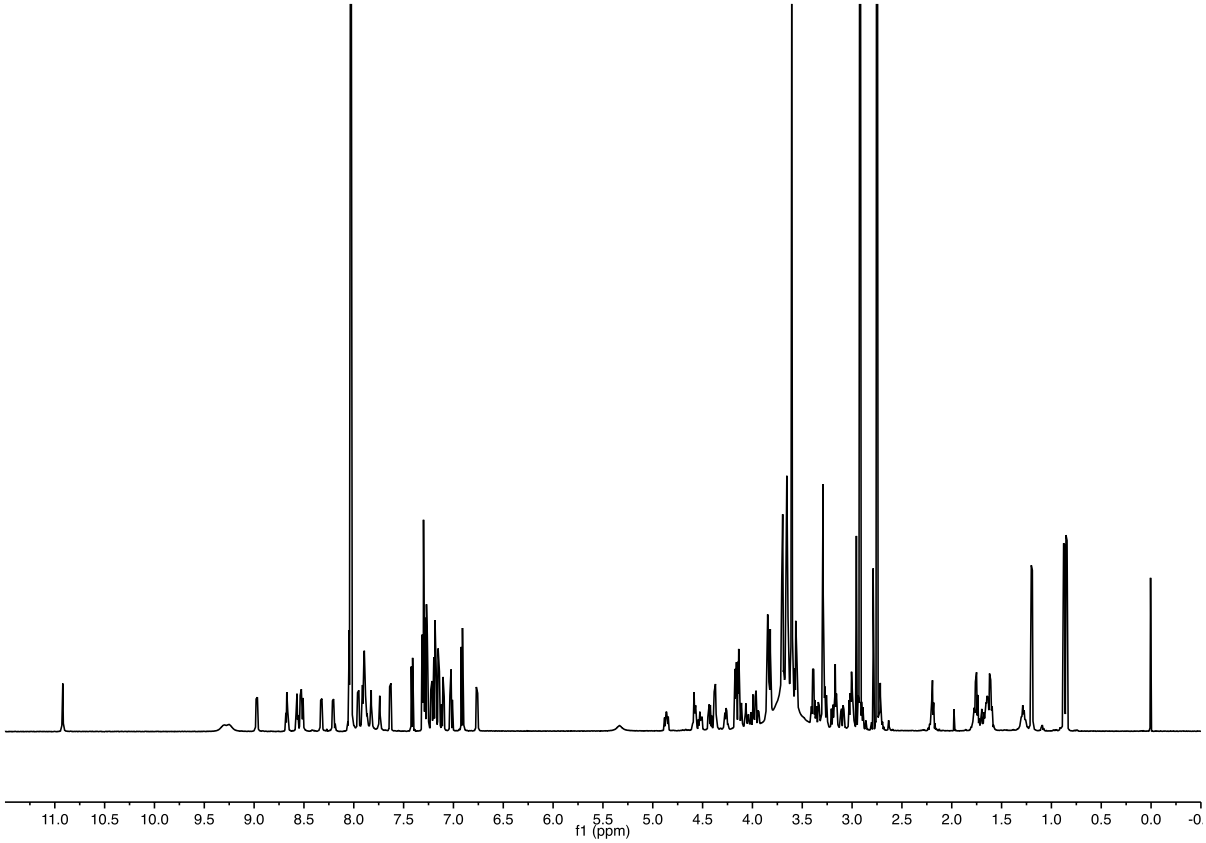


^{1}H-^{15}N HSQC (d_7-DMF)

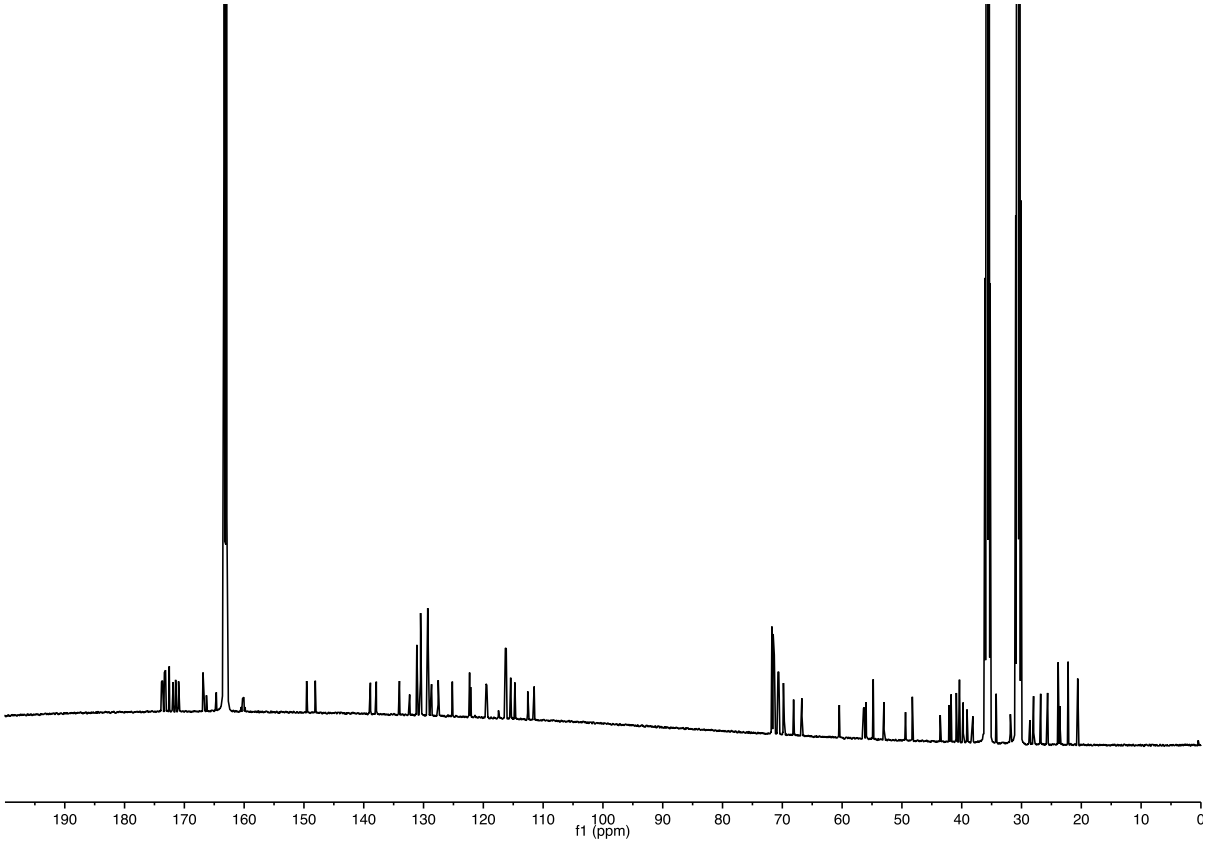


Branched-cyclic peptide B1

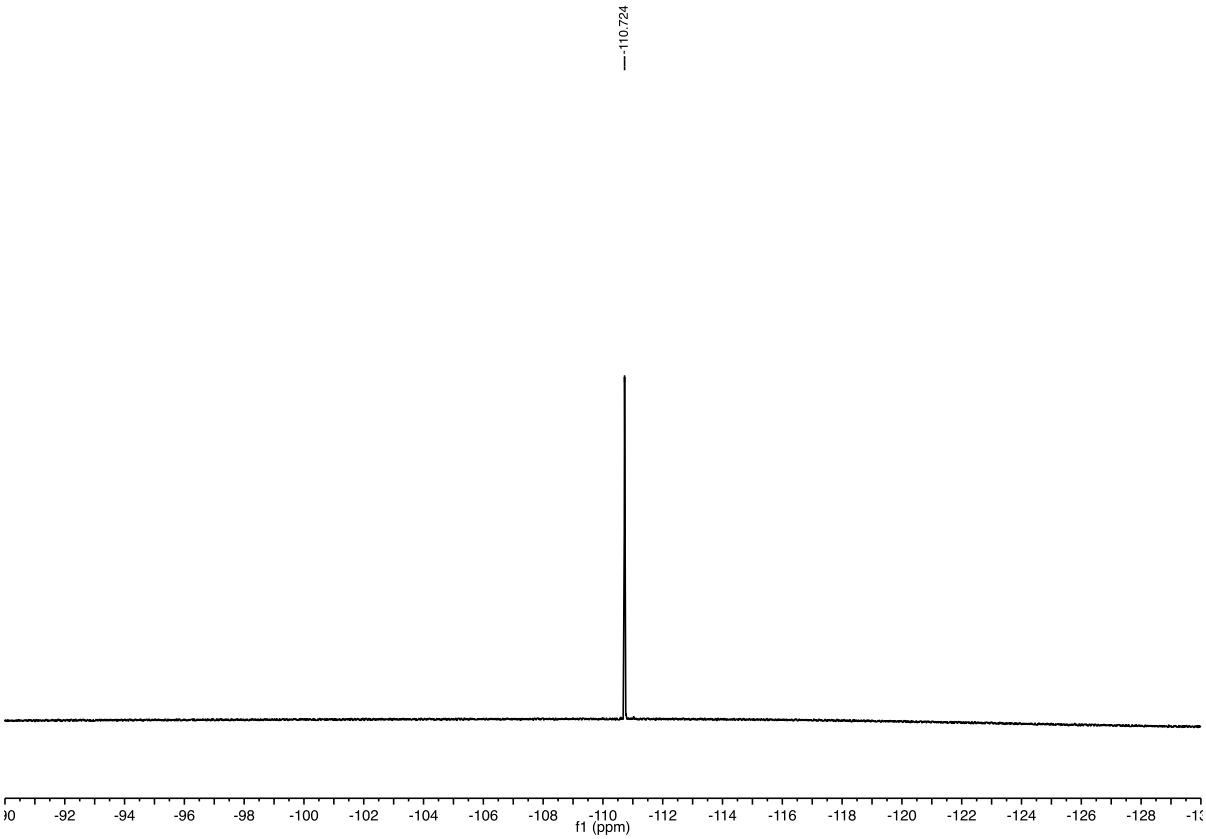
^{1}H NMR (600 MHz, d_7-DMF)



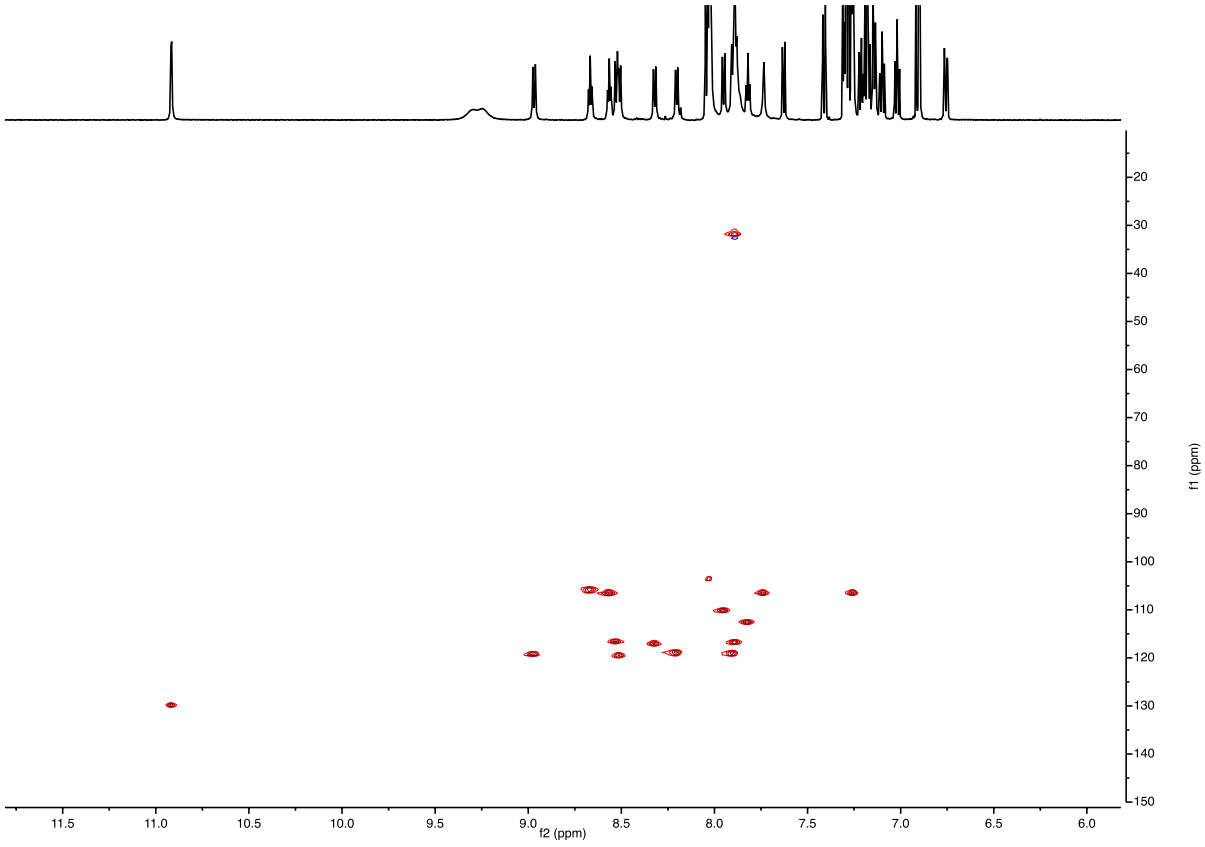
^{13}C NMR (150 MHz, d_7-DMF)



^{19}F NMR (565 MHz, d_7-DMF)

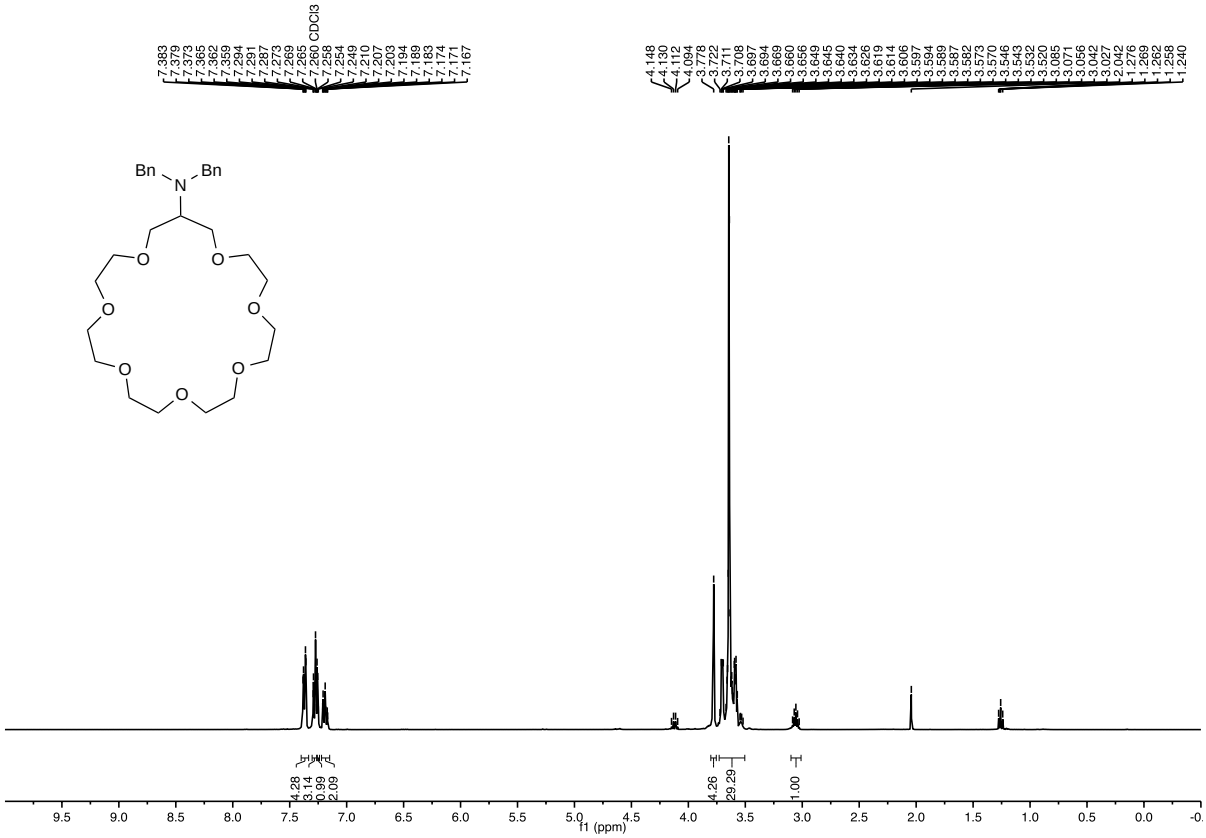


^{1}H-^{15}N HSQC (d_7-DMF)

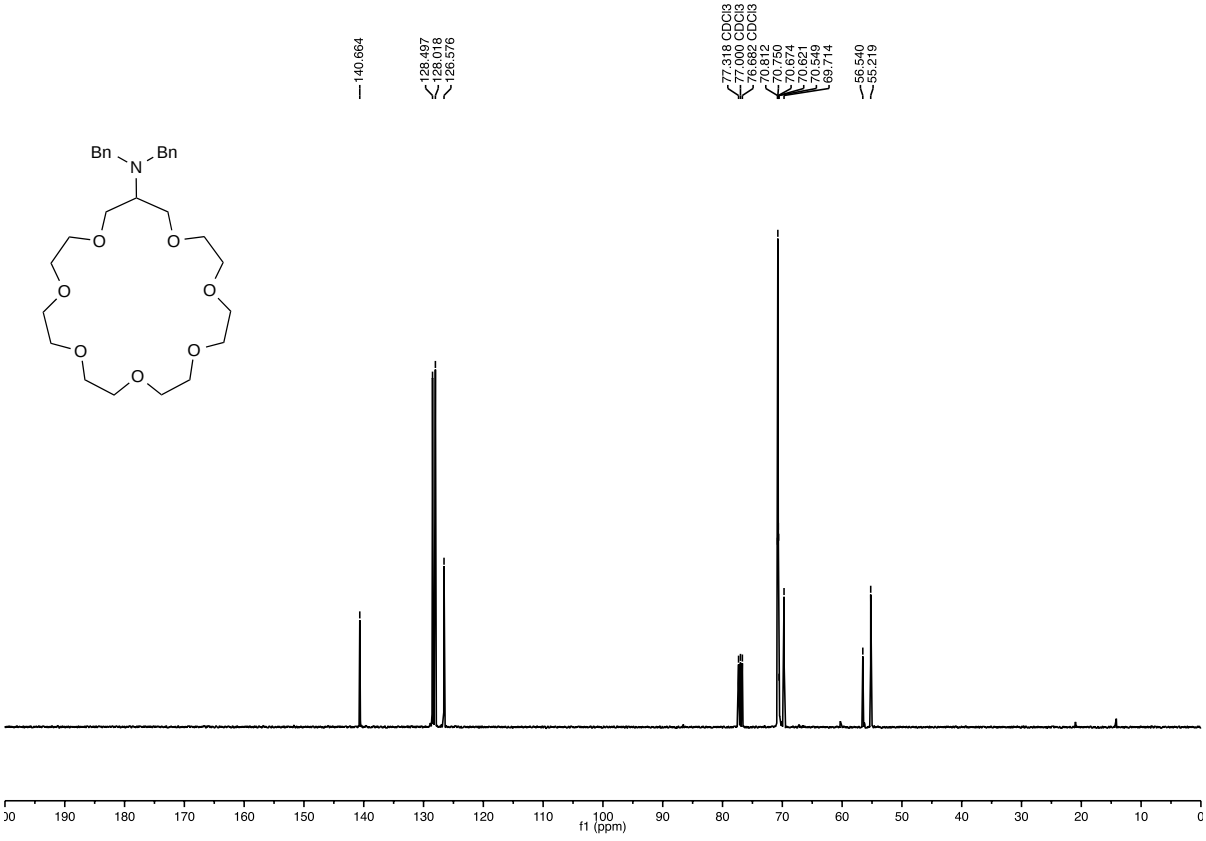


Crown ether-diBn amine S21

^{1}H NMR (400 MHz, $CDCl_3$)

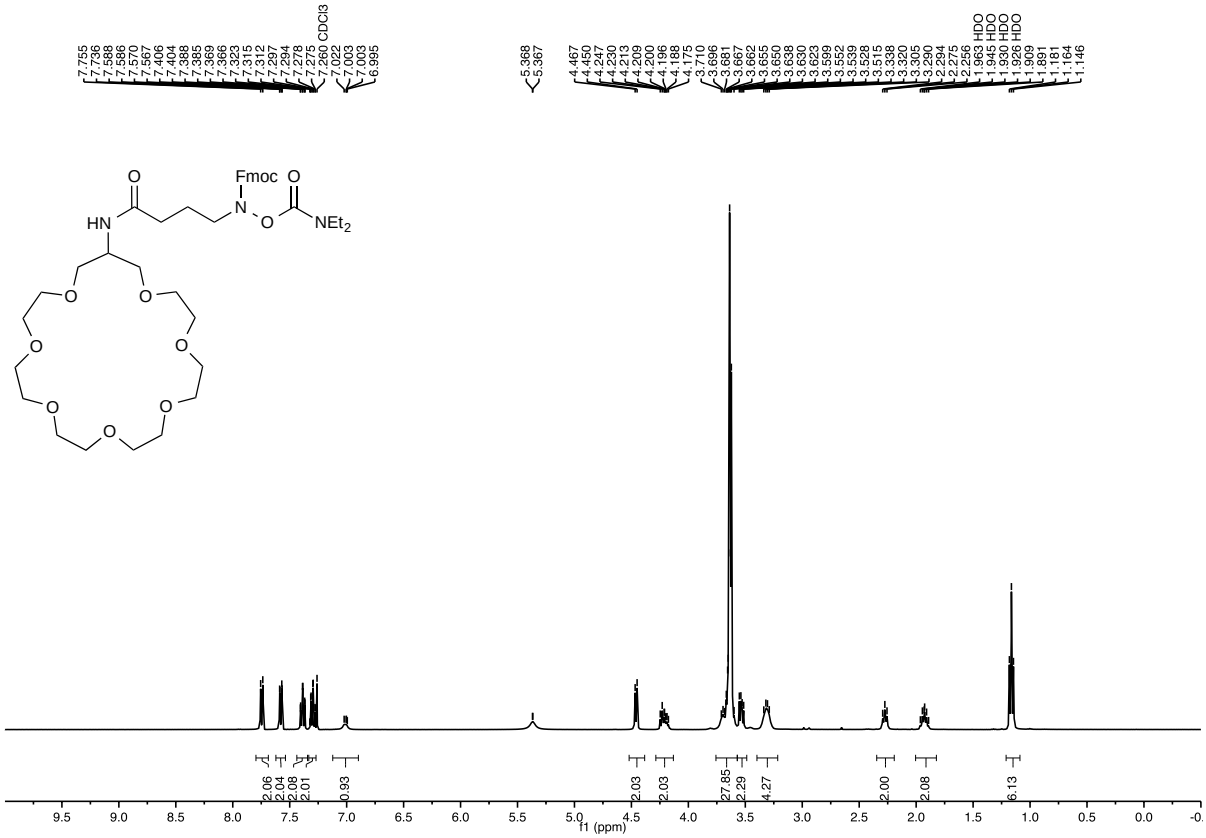


^{13}C NMR (100 MHz, $CDCl_3$)

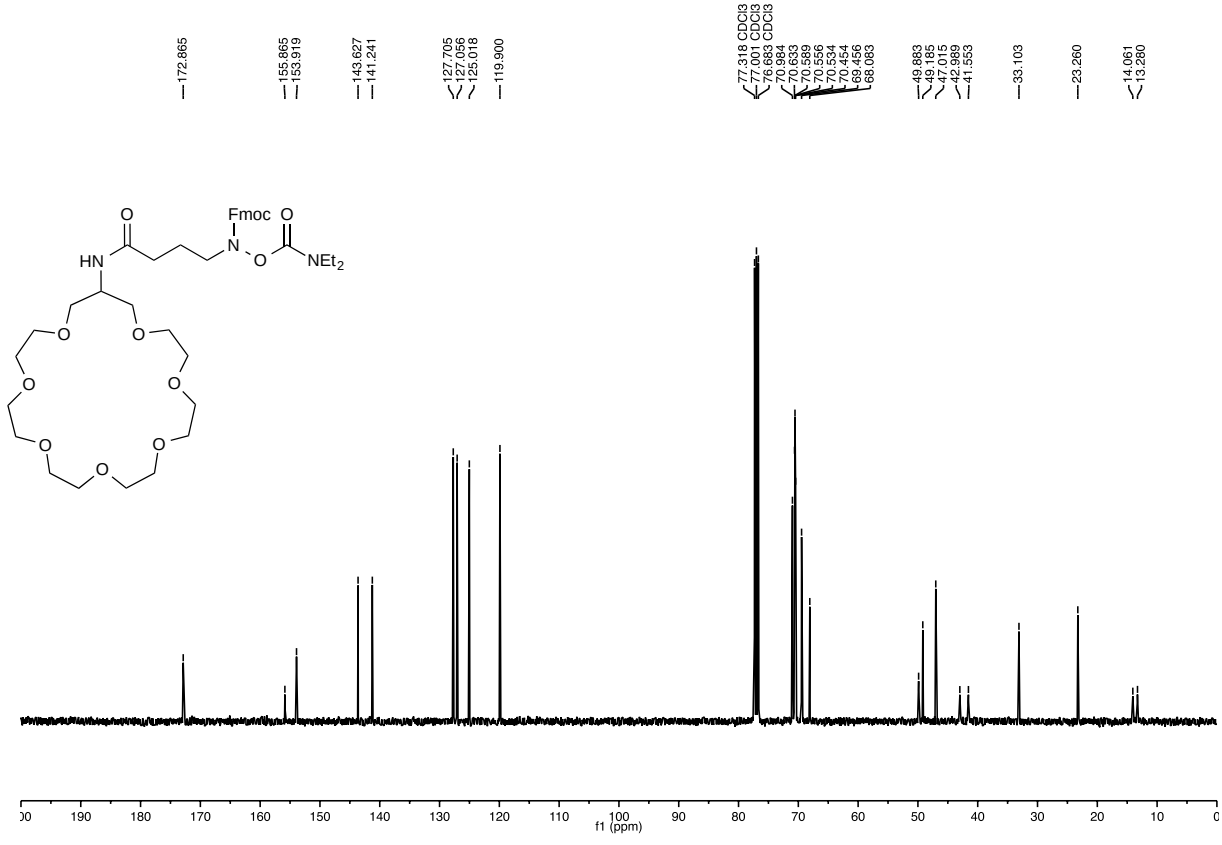


Crown ether-*N*-Fmoc hydroxylamine 13

^{1}H NMR (400 MHz, $CDCl_3$)

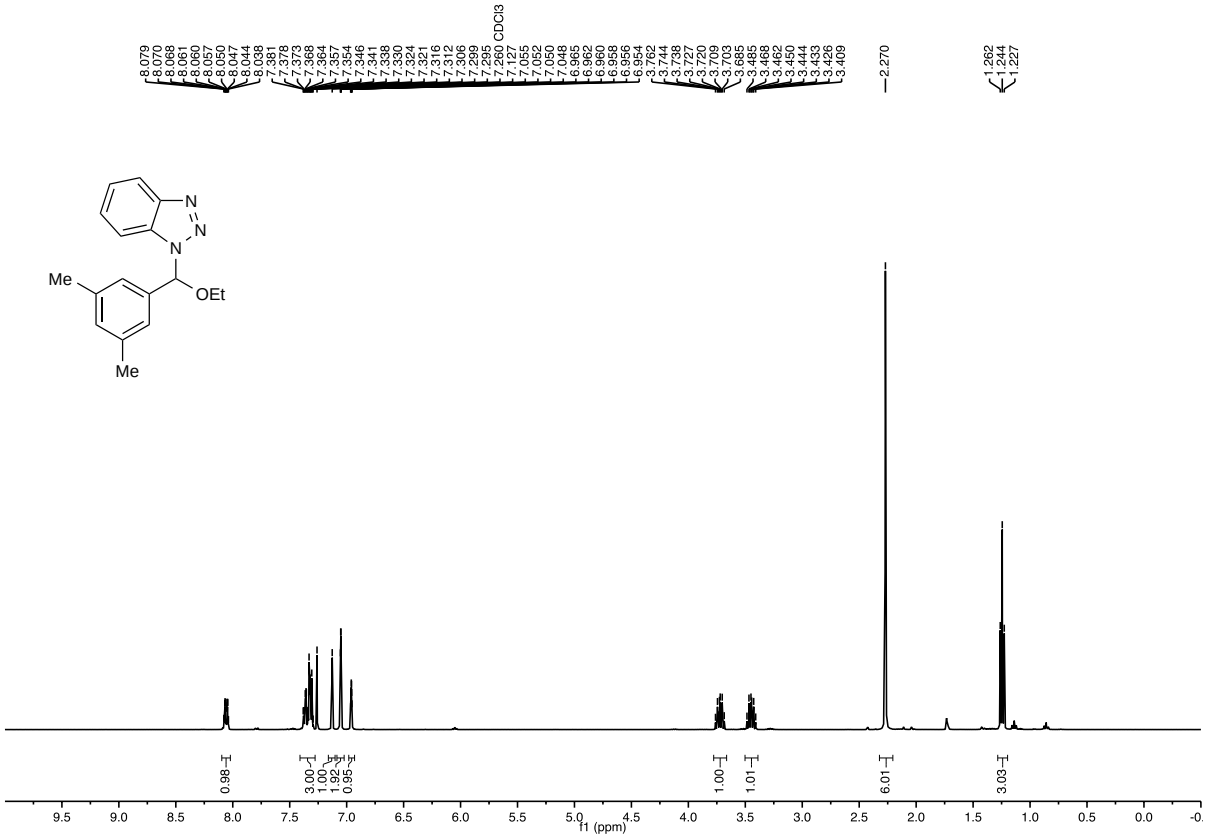


^{13}C NMR (100 MHz, $CDCl_3$)

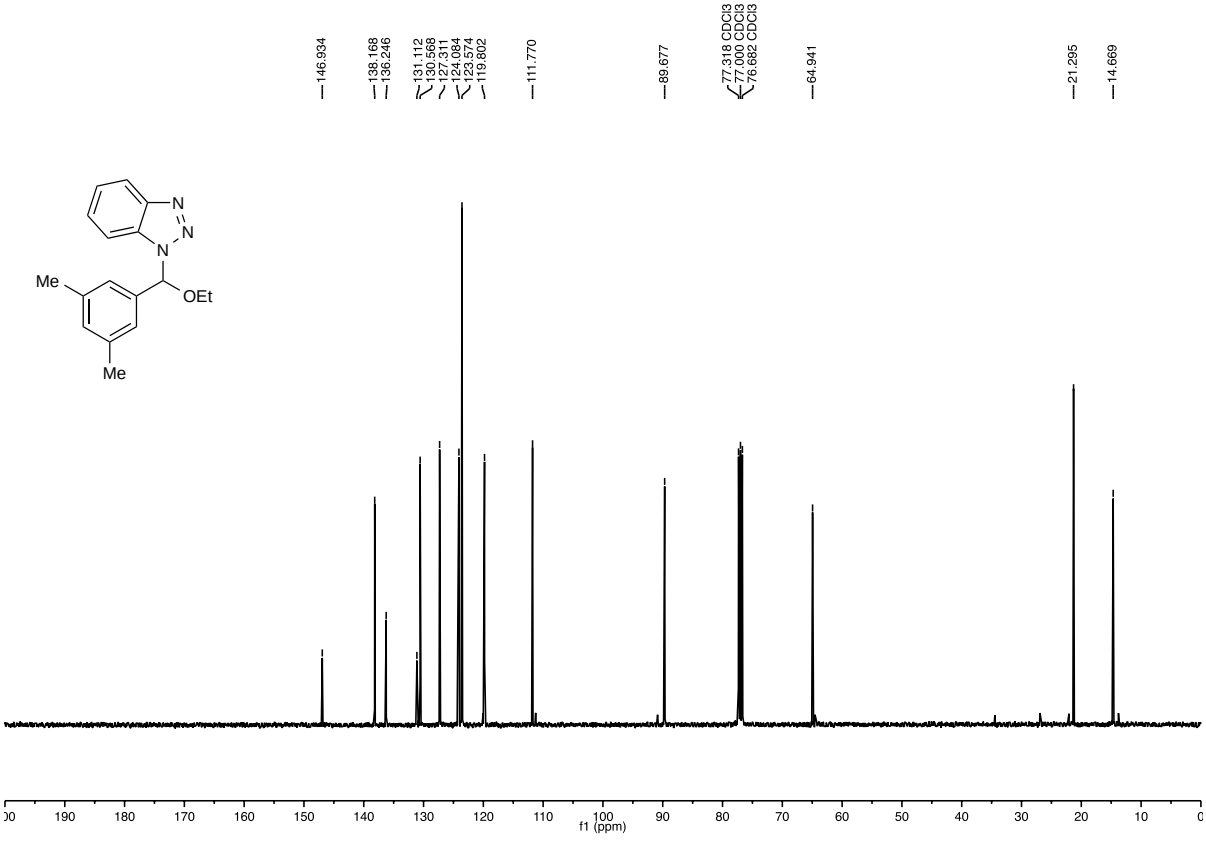


Bt-ethoxy *N,O*-acetal S22

^{1}H NMR (400 MHz, $CDCl_3$)

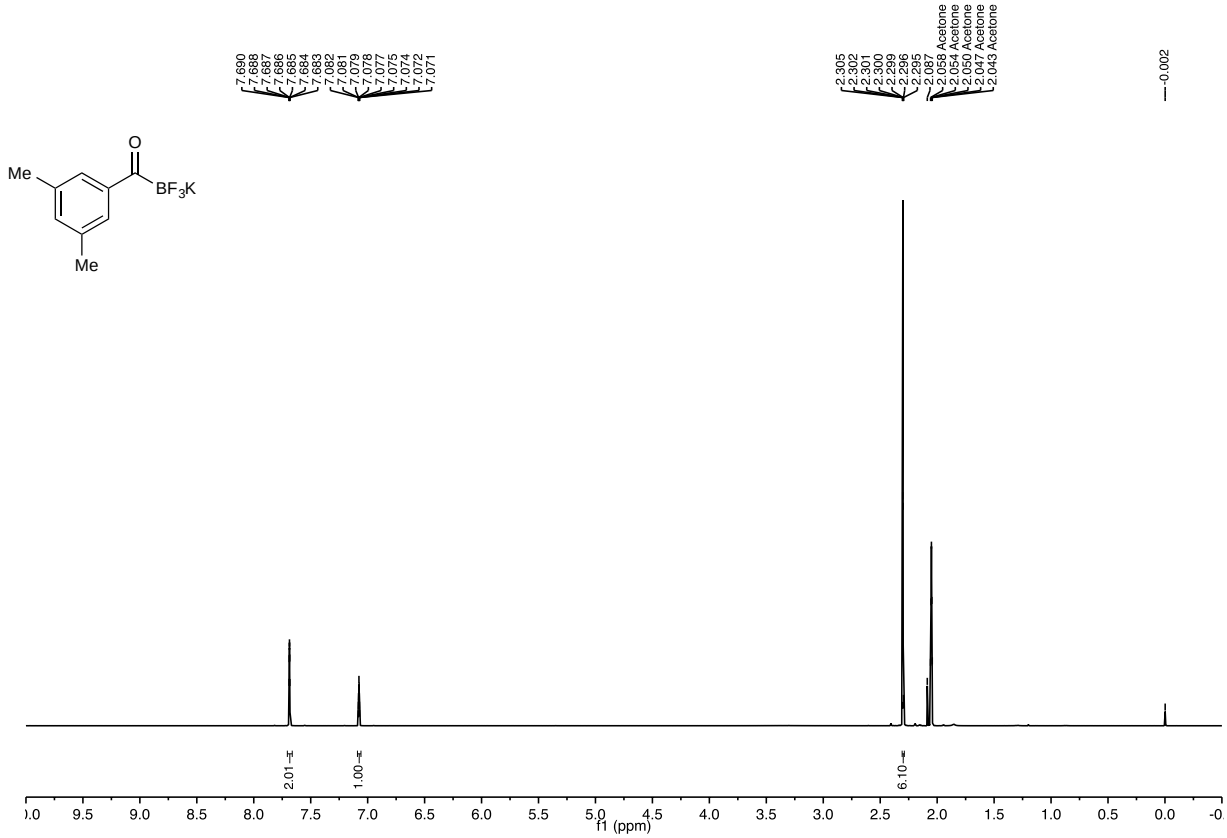


^{13}C NMR (100 MHz, $CDCl_3$)

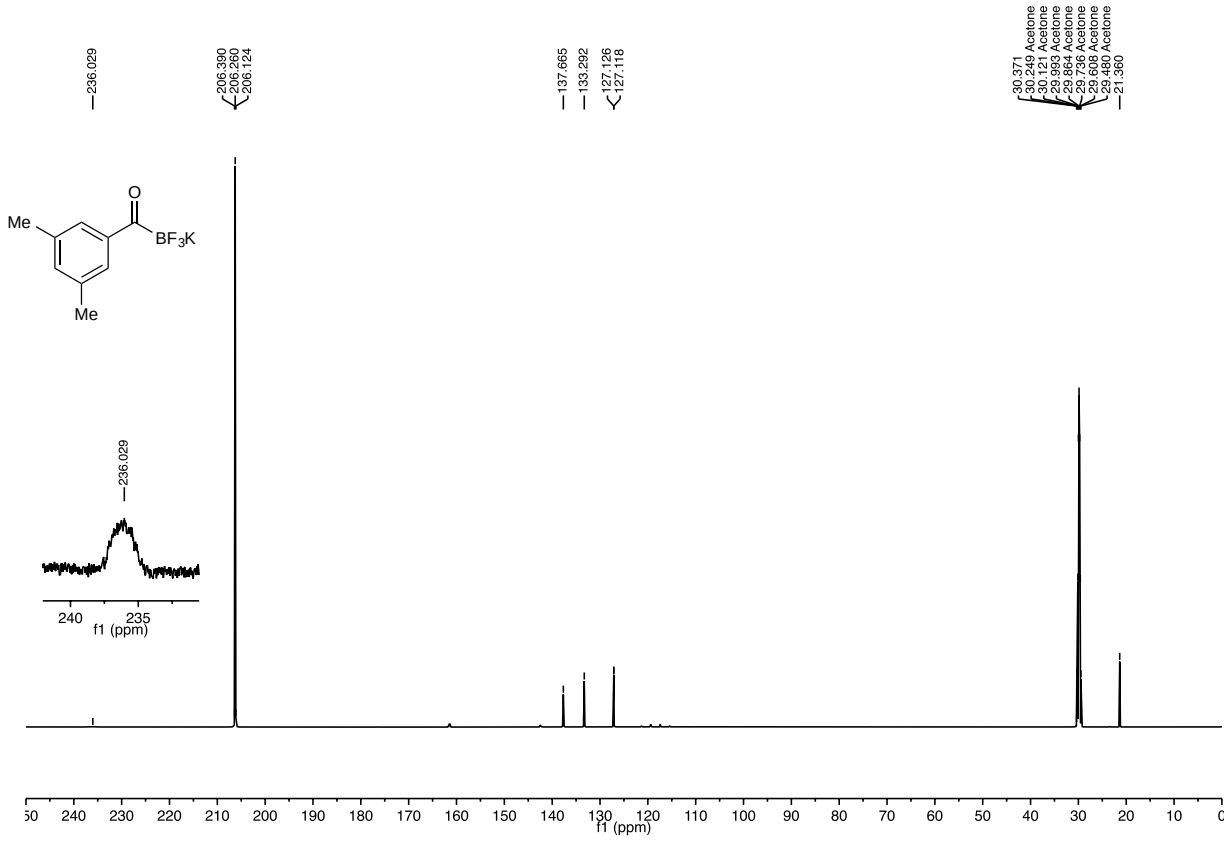


3,5-Dimethylphenyl KAT 15

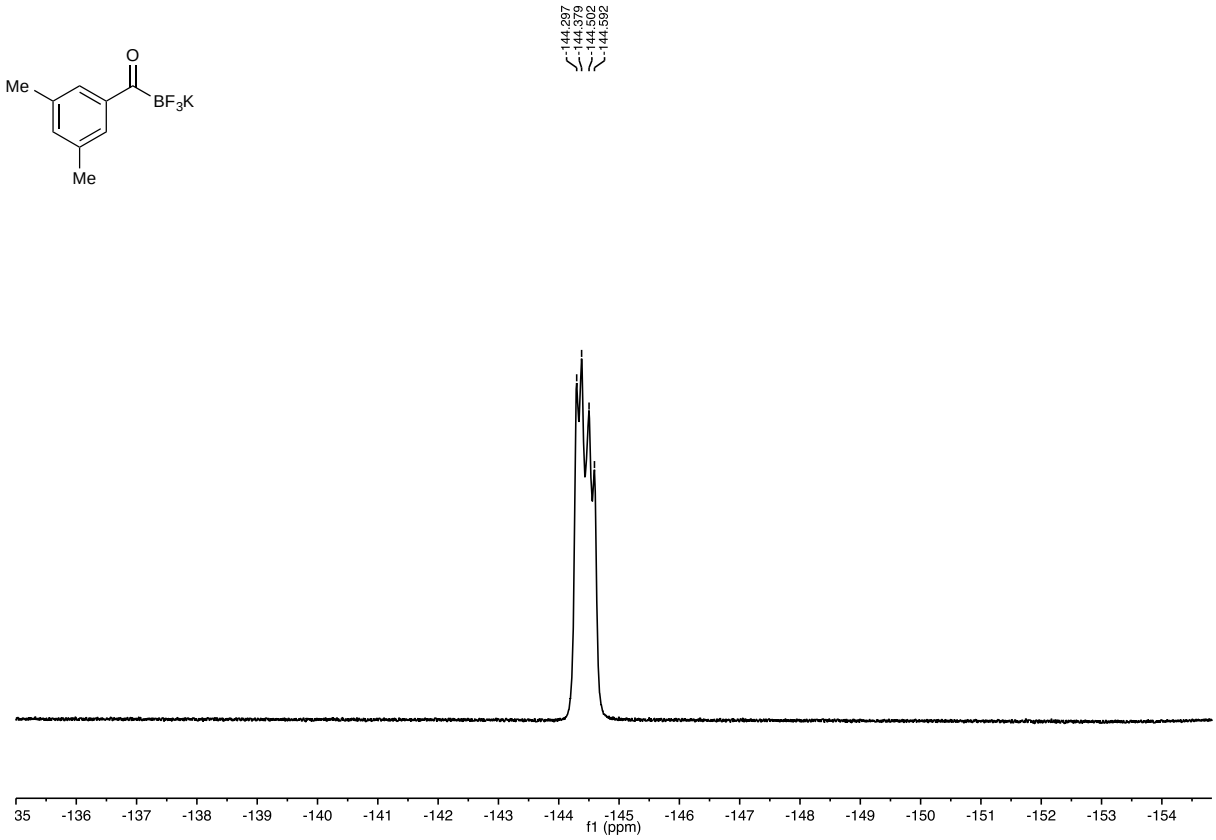
^{1}H NMR (600 MHz, d_6-acetone)



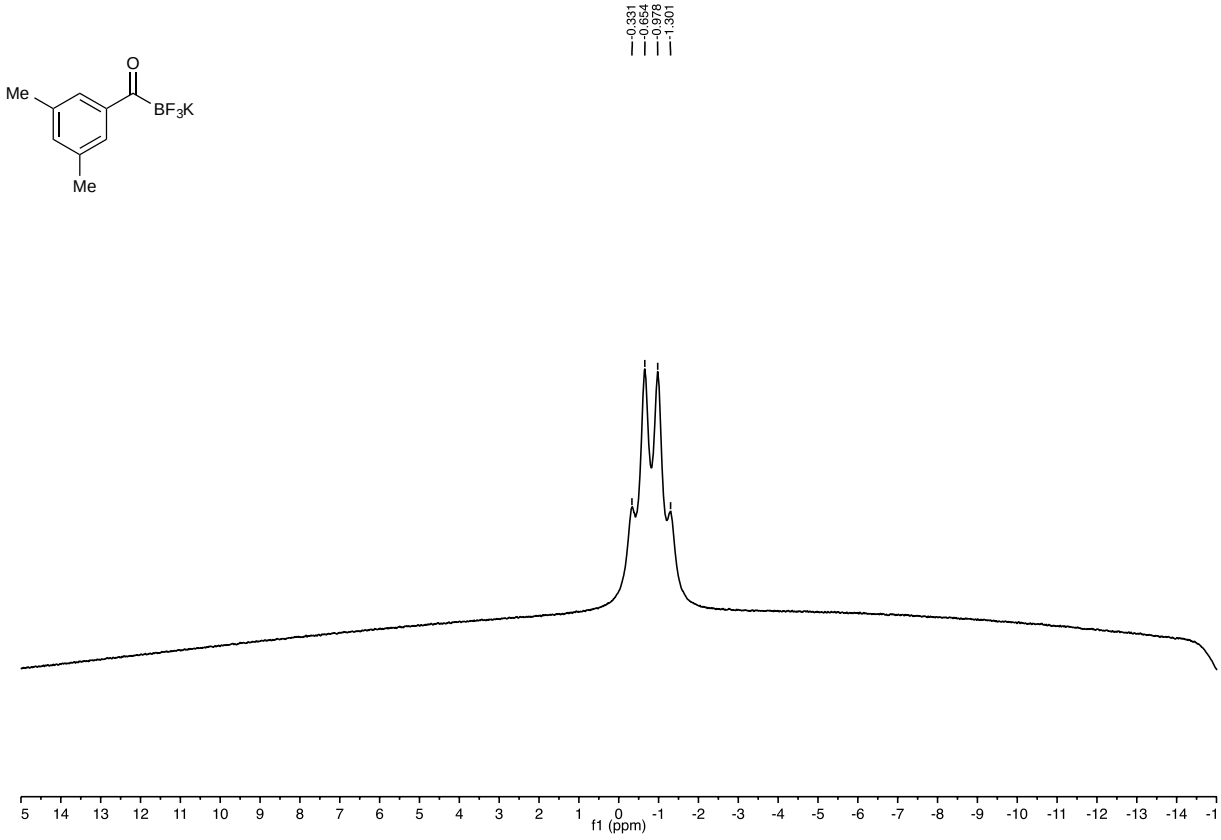
^{13}C NMR (150 MHz, d_6-acetone)



^{19}F NMR (470 MHz, d_6-acetone)

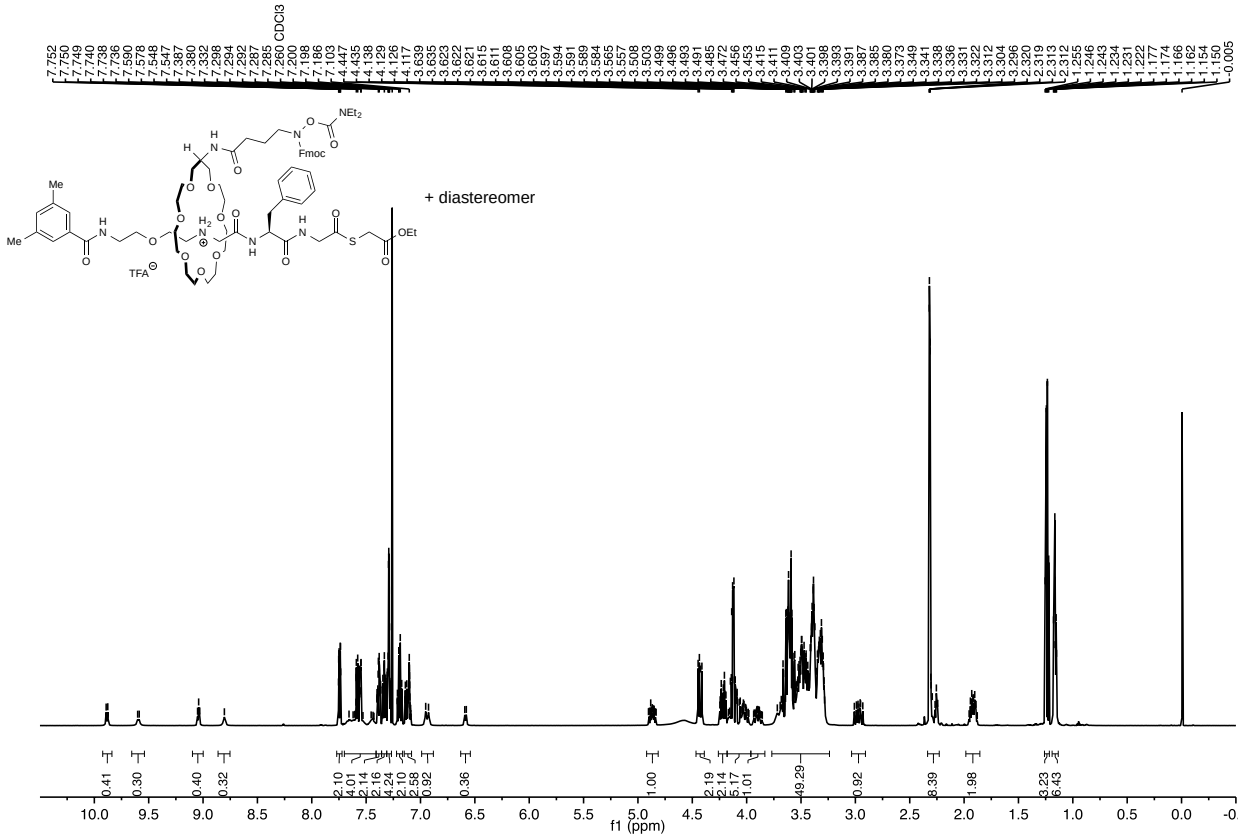


^{11}B NMR (160 MHz, d_6-acetone)

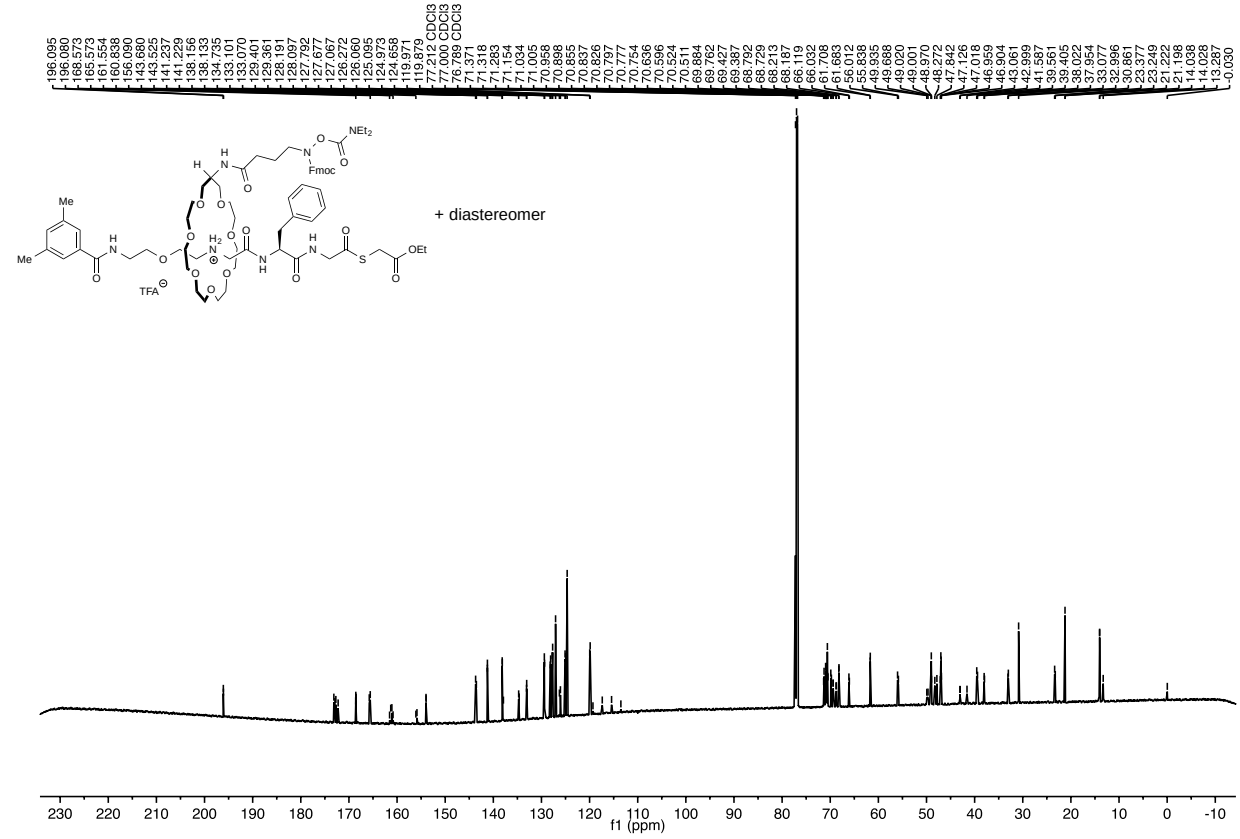


[2]Rotaxane S23

^{1}H NMR (600 MHz, $CDCl_3$)

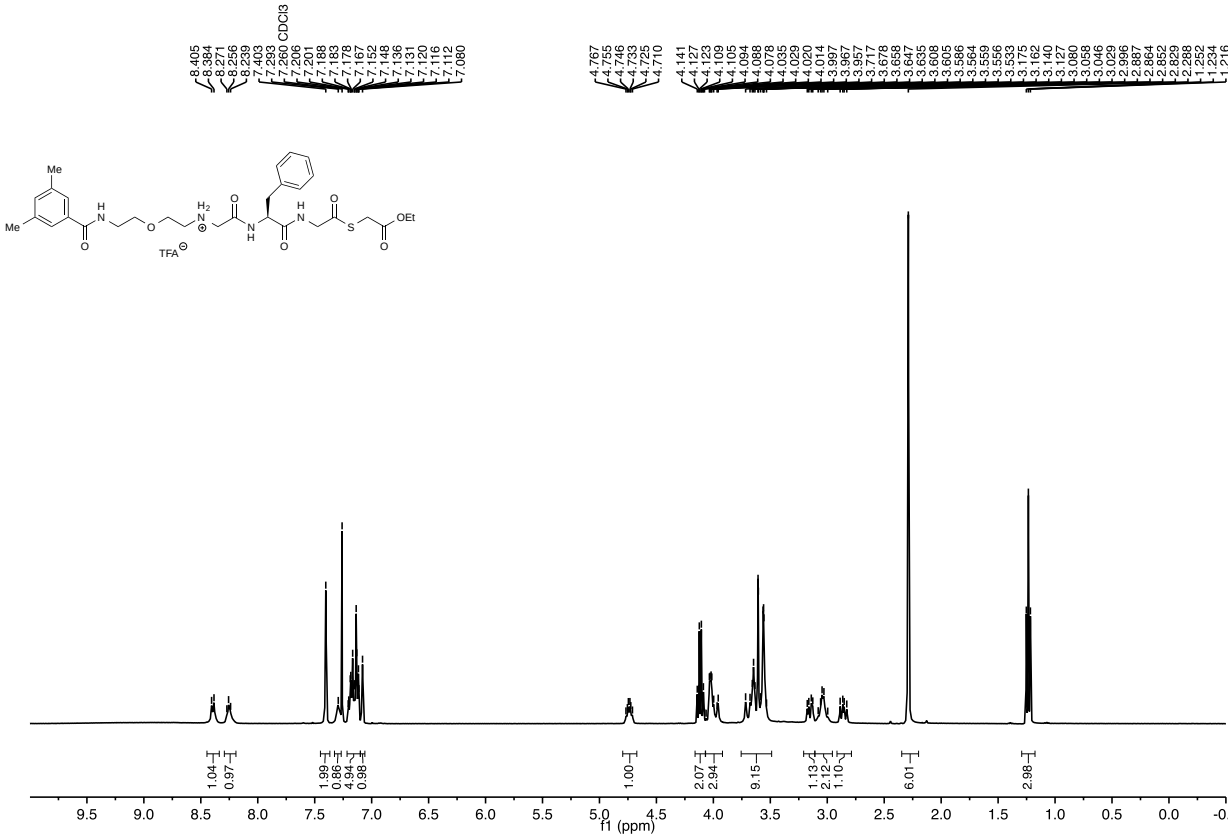


^{13}C NMR (150 MHz, $CDCl_3$)

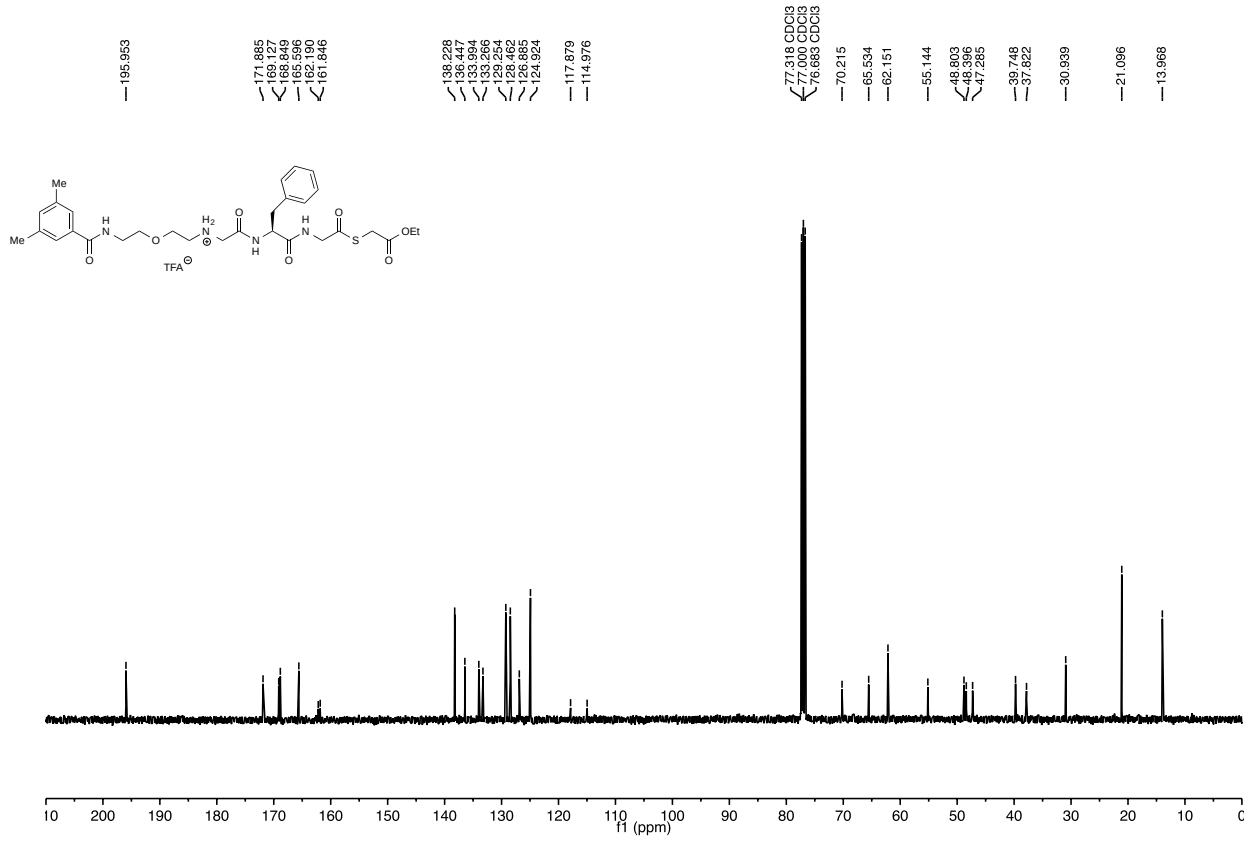


Axle S24

^{1}H NMR (400 MHz, $CDCl_3$)

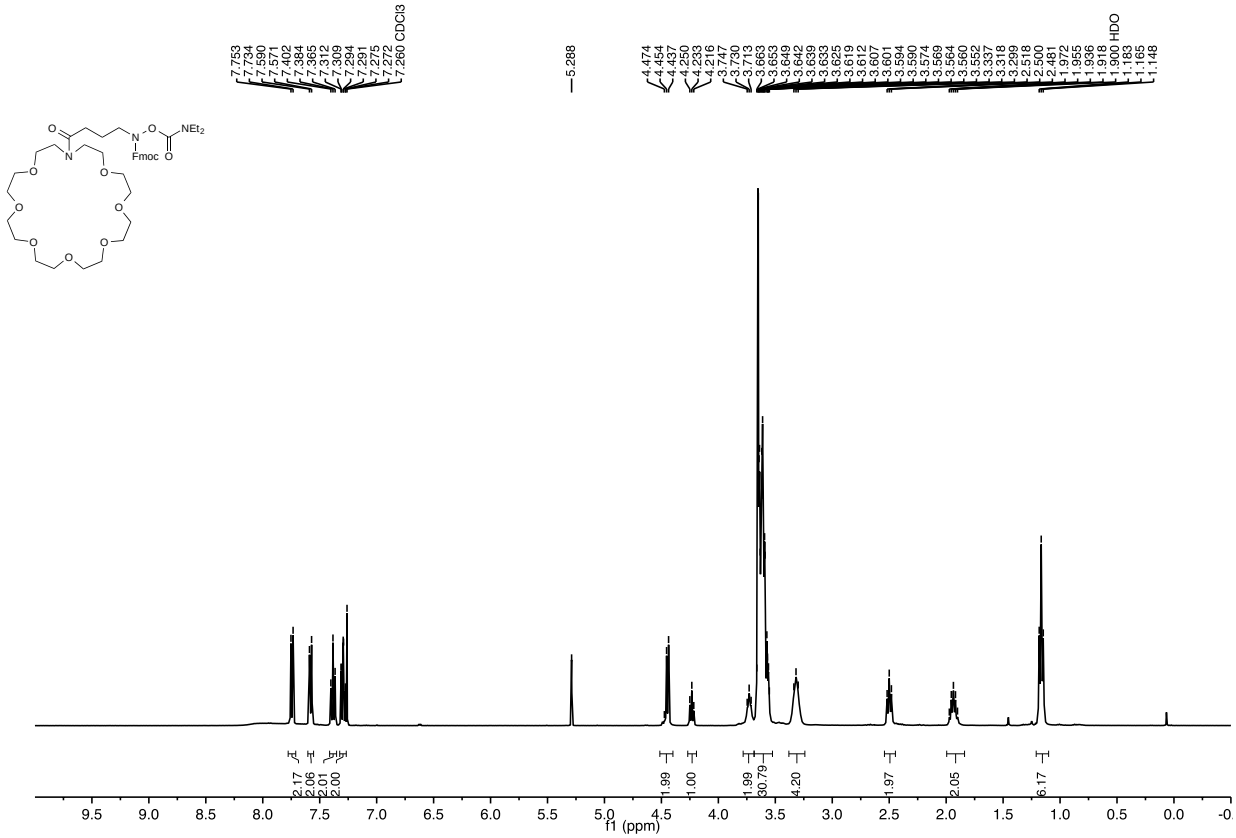


^{13}C NMR (100 MHz, $CDCl_3$)

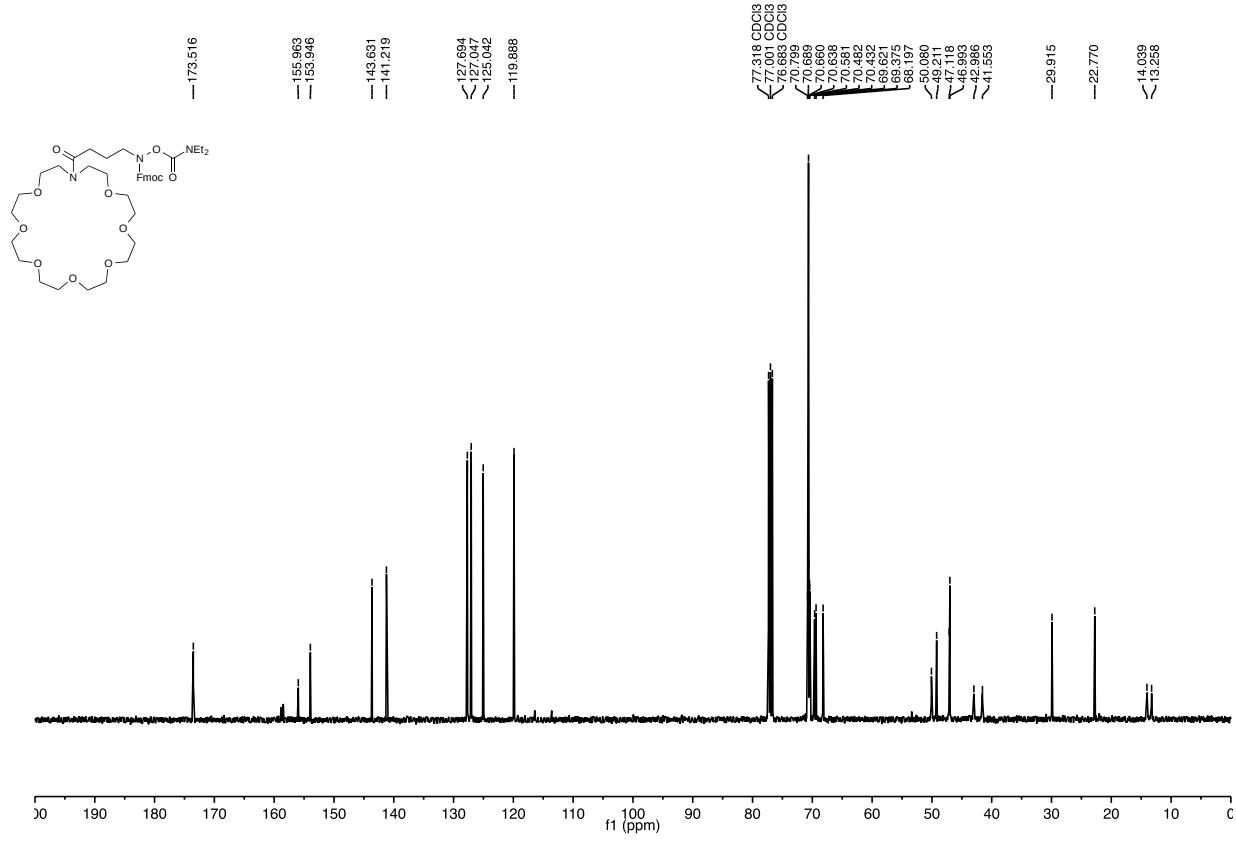


Crown ether *N*-Fmoc hydroxylamine 14

^{1}H NMR (400 MHz, $CDCl_3$)

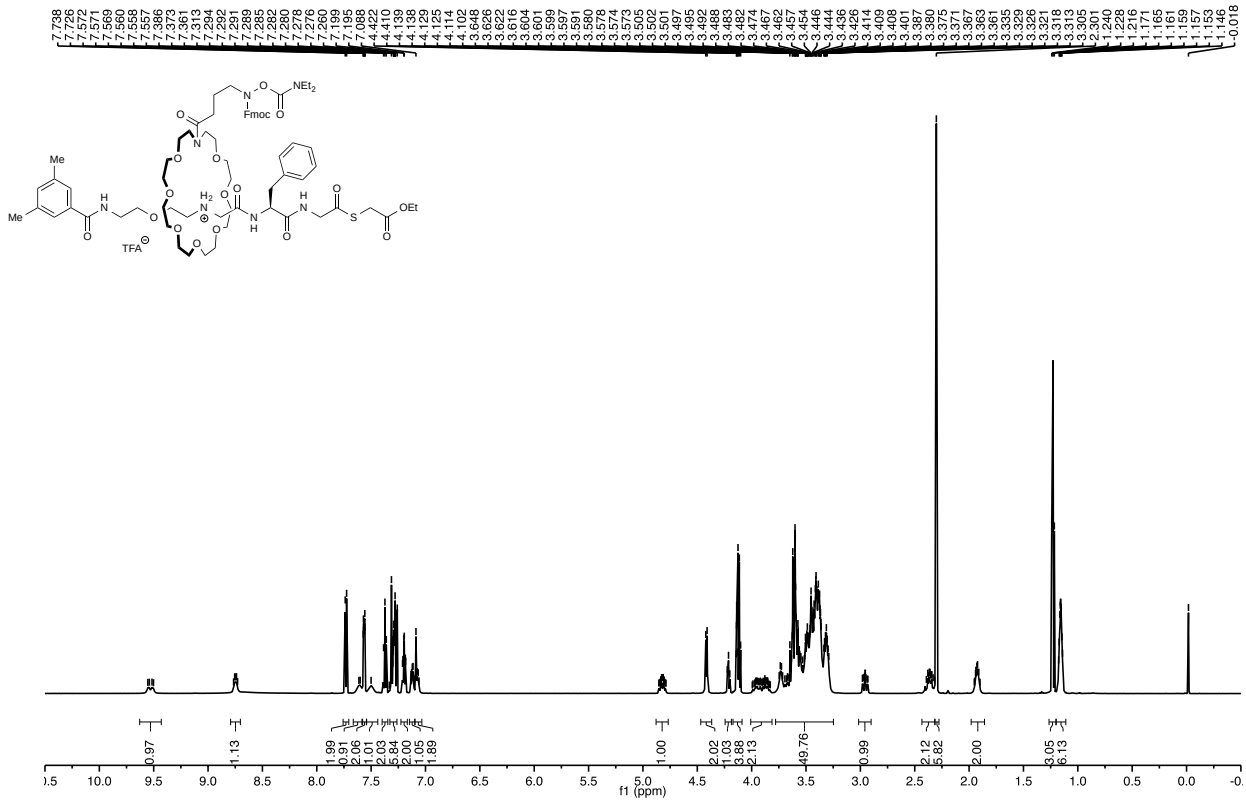


^{13}C NMR (100 MHz, $CDCl_3$)

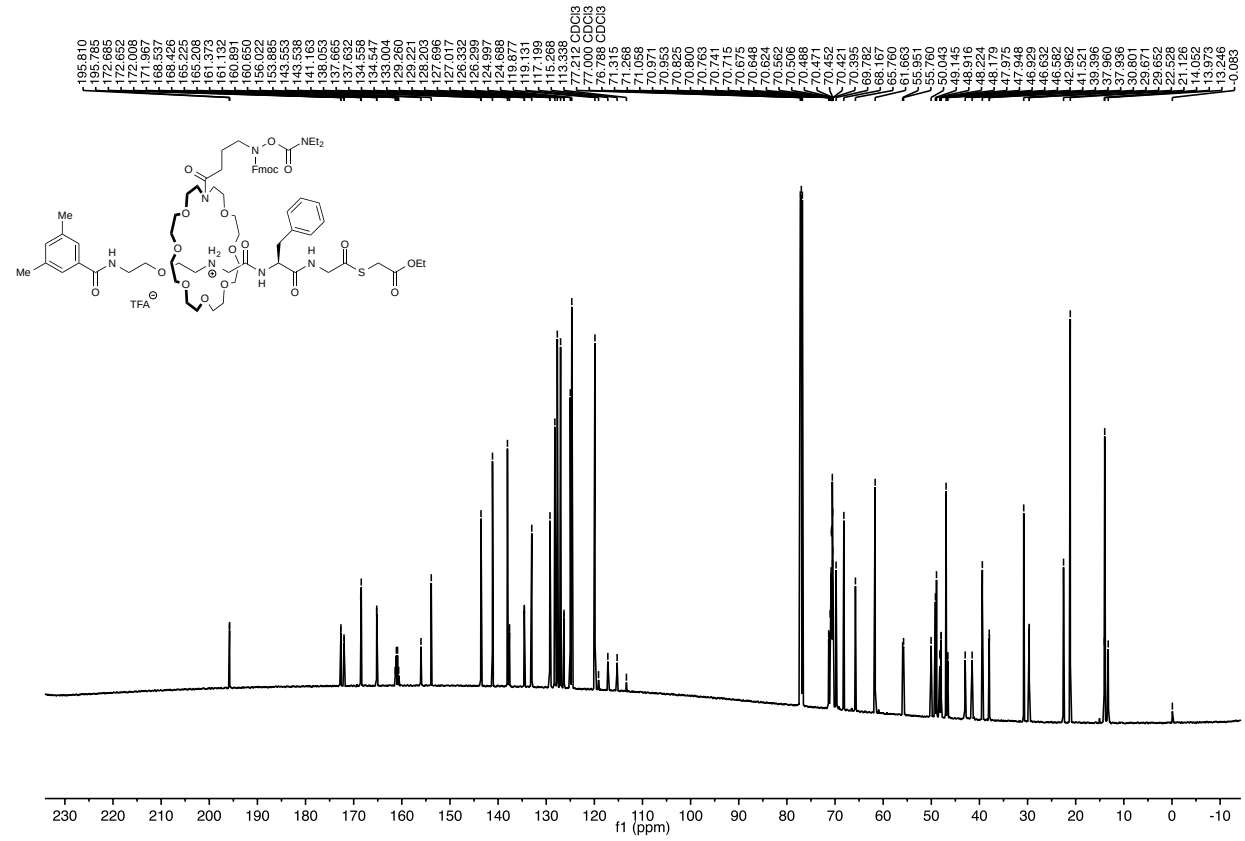


[2]Rotaxane S31

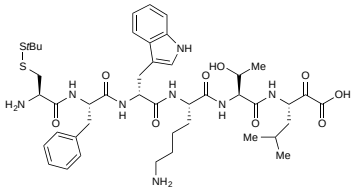
^{1}H NMR (600 MHz, $CDCl_3$)



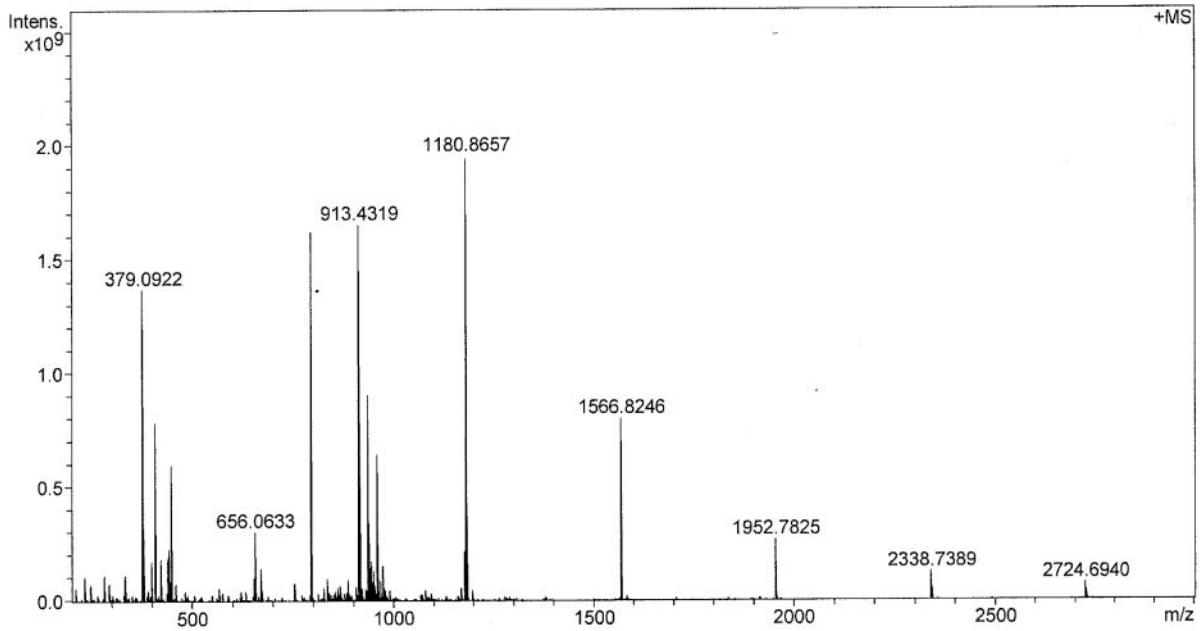
^{13}C NMR (150 MHz, $CDCl_3$)

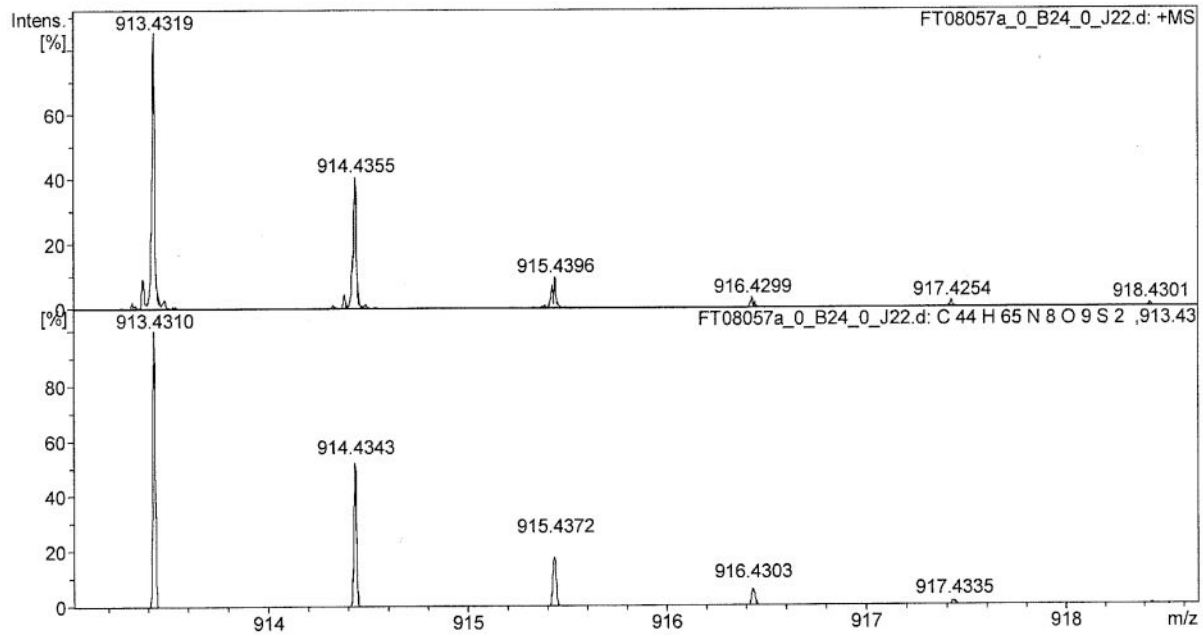


Peptide α-ketoacid 7

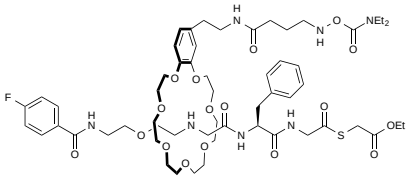


Box 1: Full scale spectra with internal reference peaks (ESI: Na-PFHA 408.9481, 794.9069, 1180.8657, 1566.8246); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.

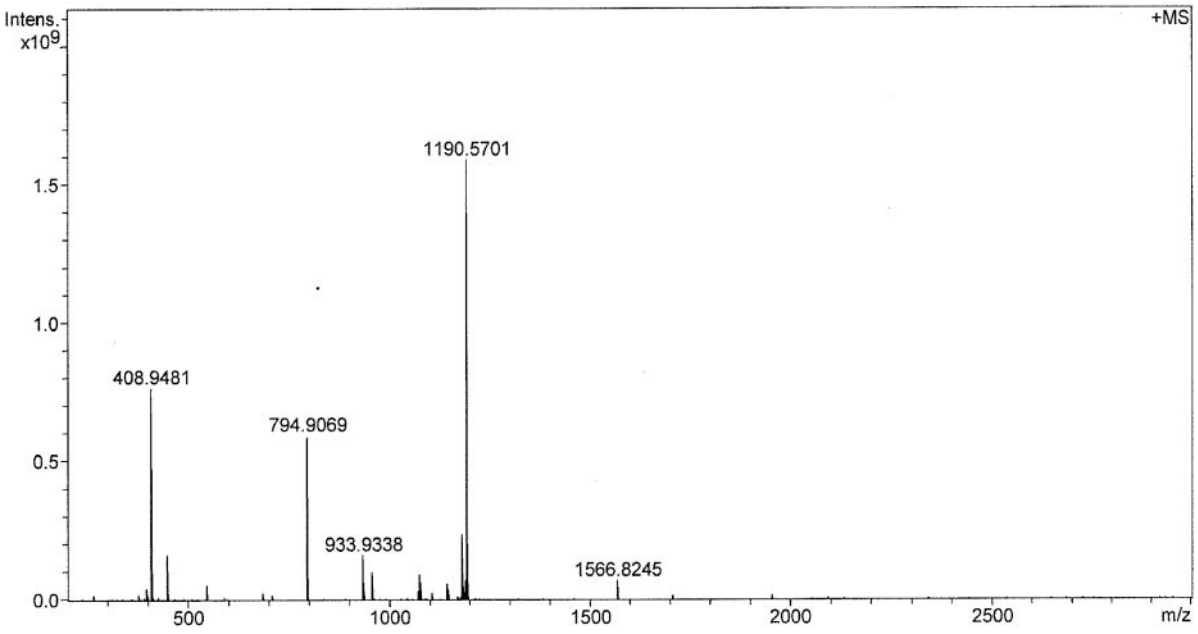


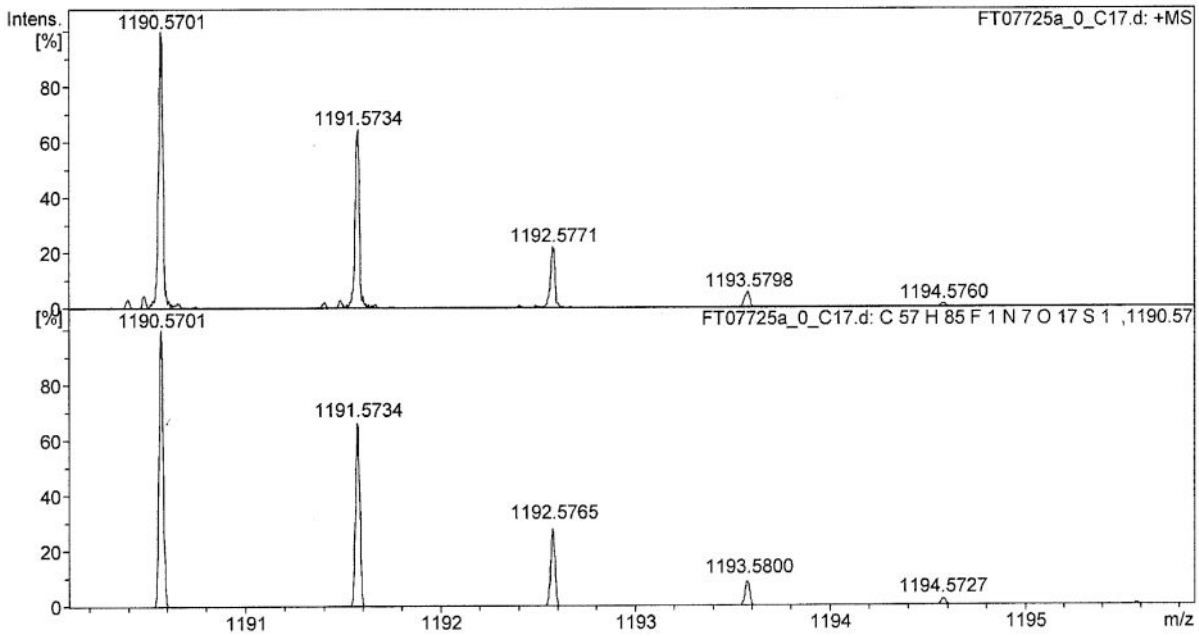


Unprotected hydroxylamine 6

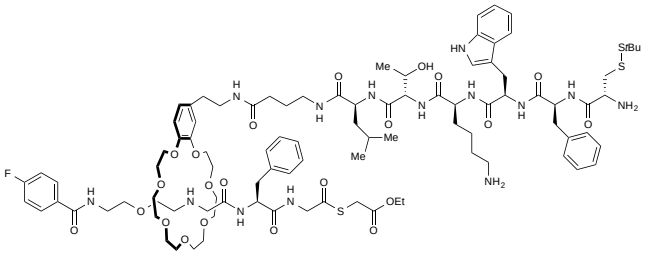


Box 1: Full scale spectra with internal reference peaks (ESI: Na-PFHA 408.9481, 794.9069, 1180.8657, 1566.8246); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.

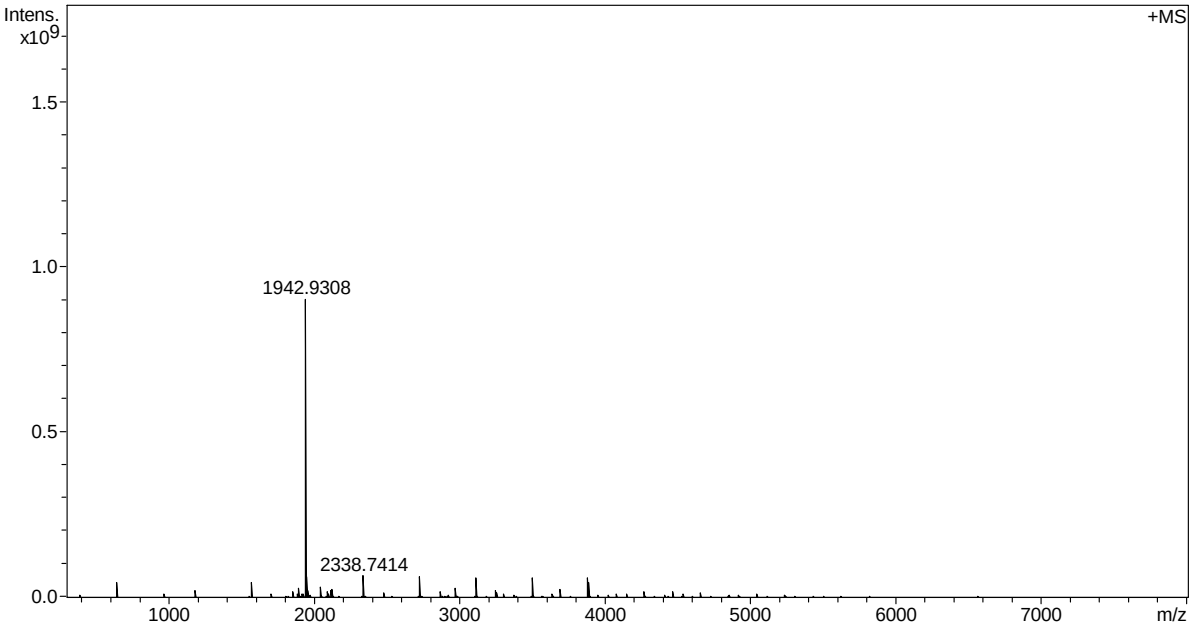


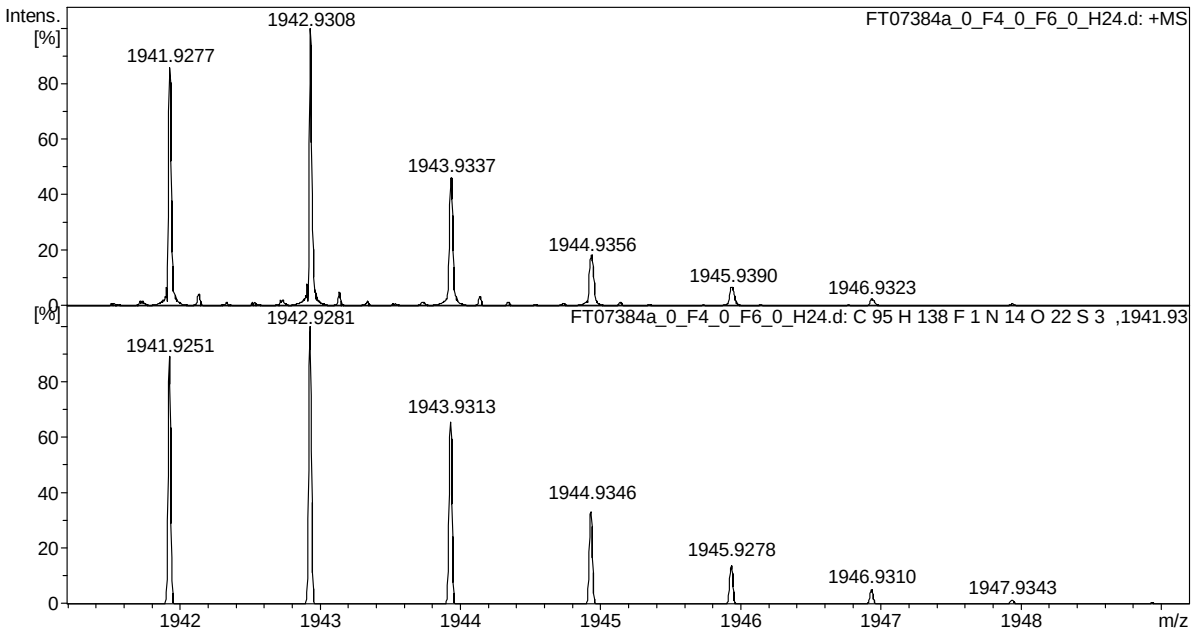


Peptido[2]rotaxane 8

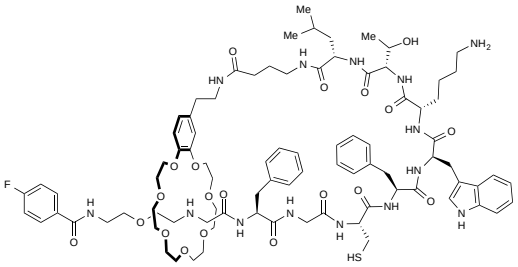


Box 1: Full scale spectra with internal reference peaks (ESI: Na-PFHA 1566.8246, 1952.7834, 2338.7423, 3110.6599); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.

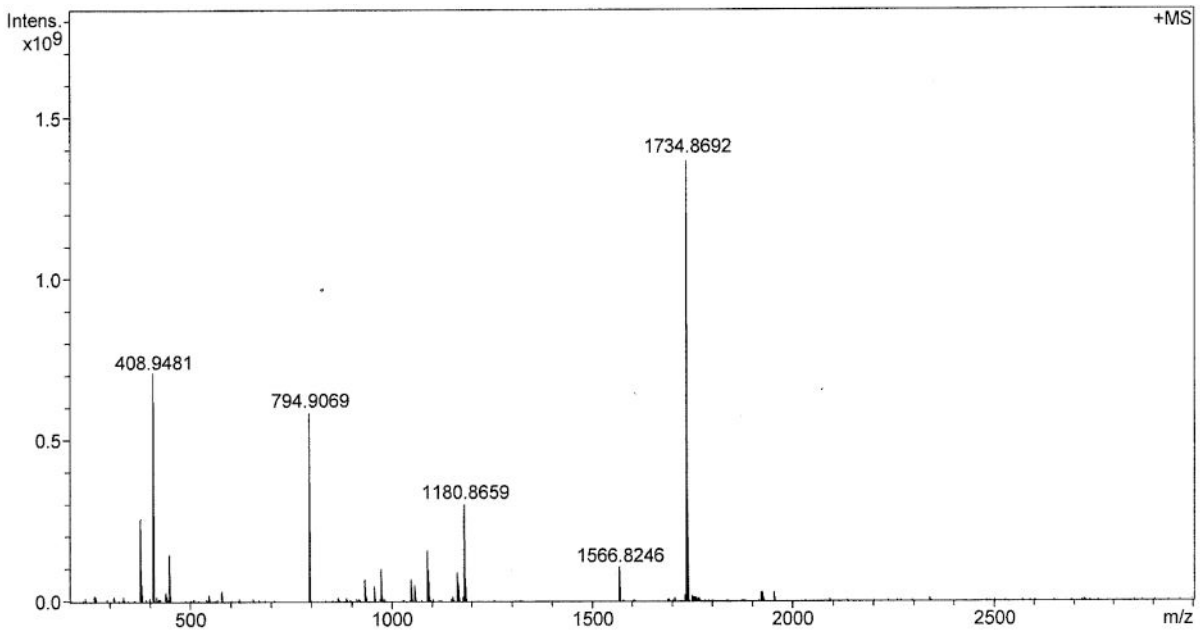


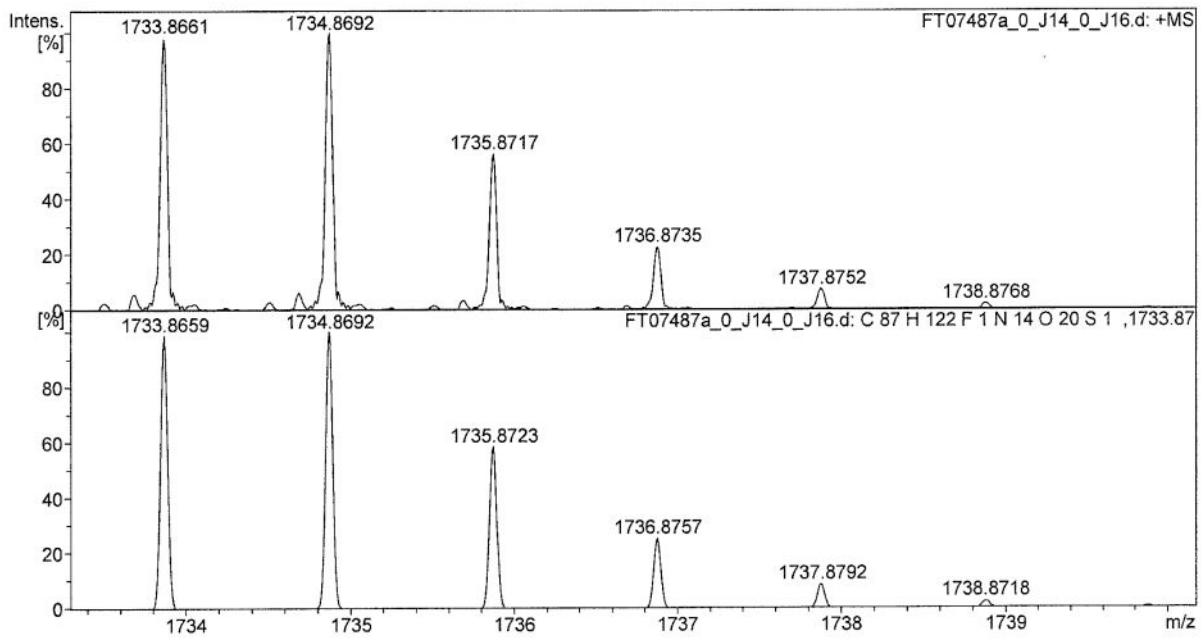


Lasso peptide 9

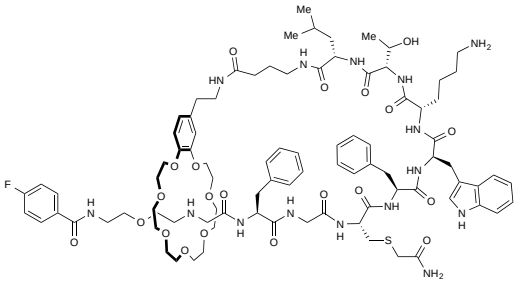


Box 1: Full scale spectra with internal reference peaks (ESI: Na-PFHA 408.9481, 794.9069, 1180.8657, 1566.8246, 1952.7834); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.

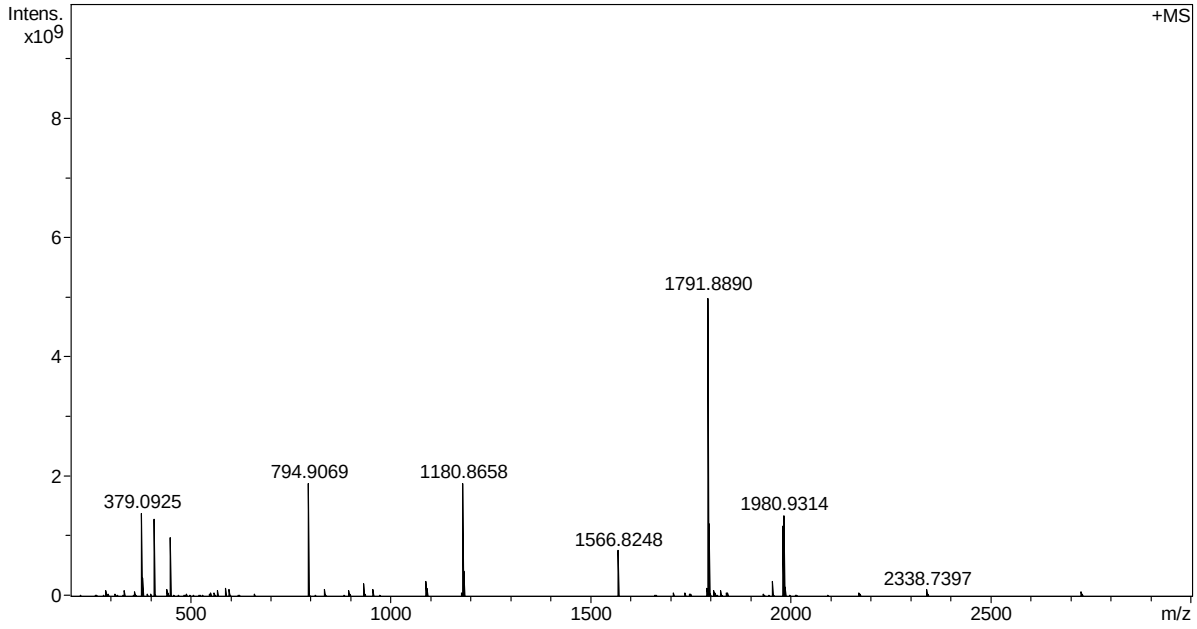


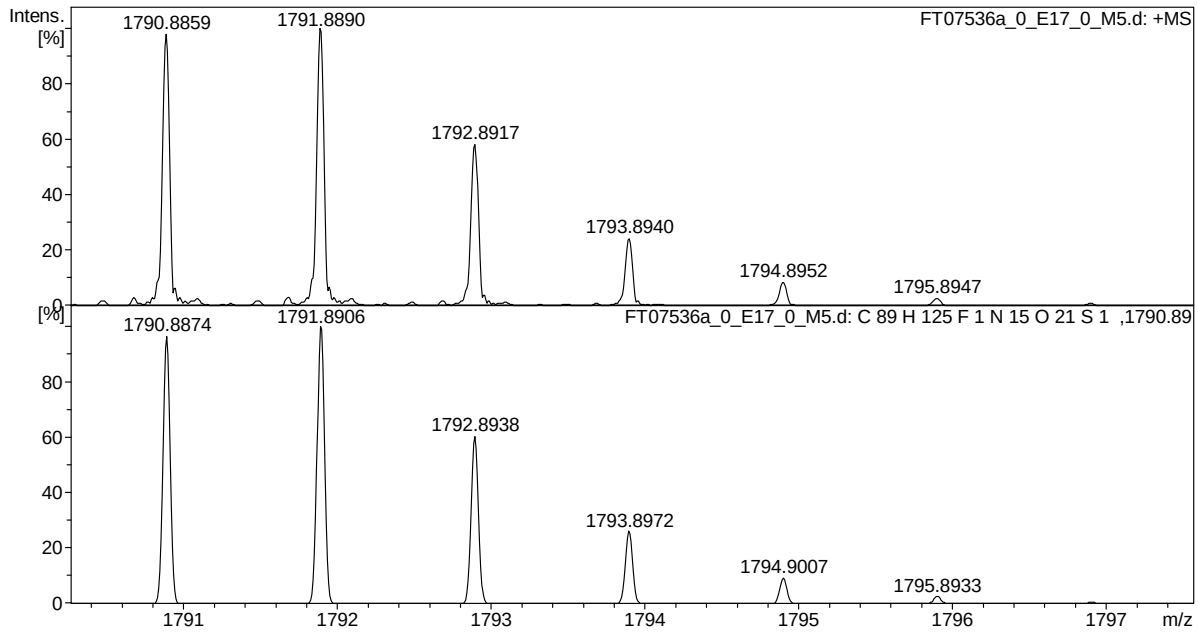


Cys-alkylated lasso peptide L1

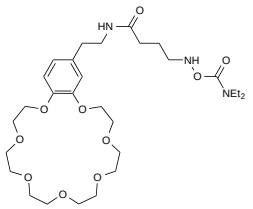


Box 1: Full scale spectra with internal reference peaks (ESI: Na-PFHA 408.9481, 794.9069, 1180.8657, 1566.8246, 1952.7834); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.

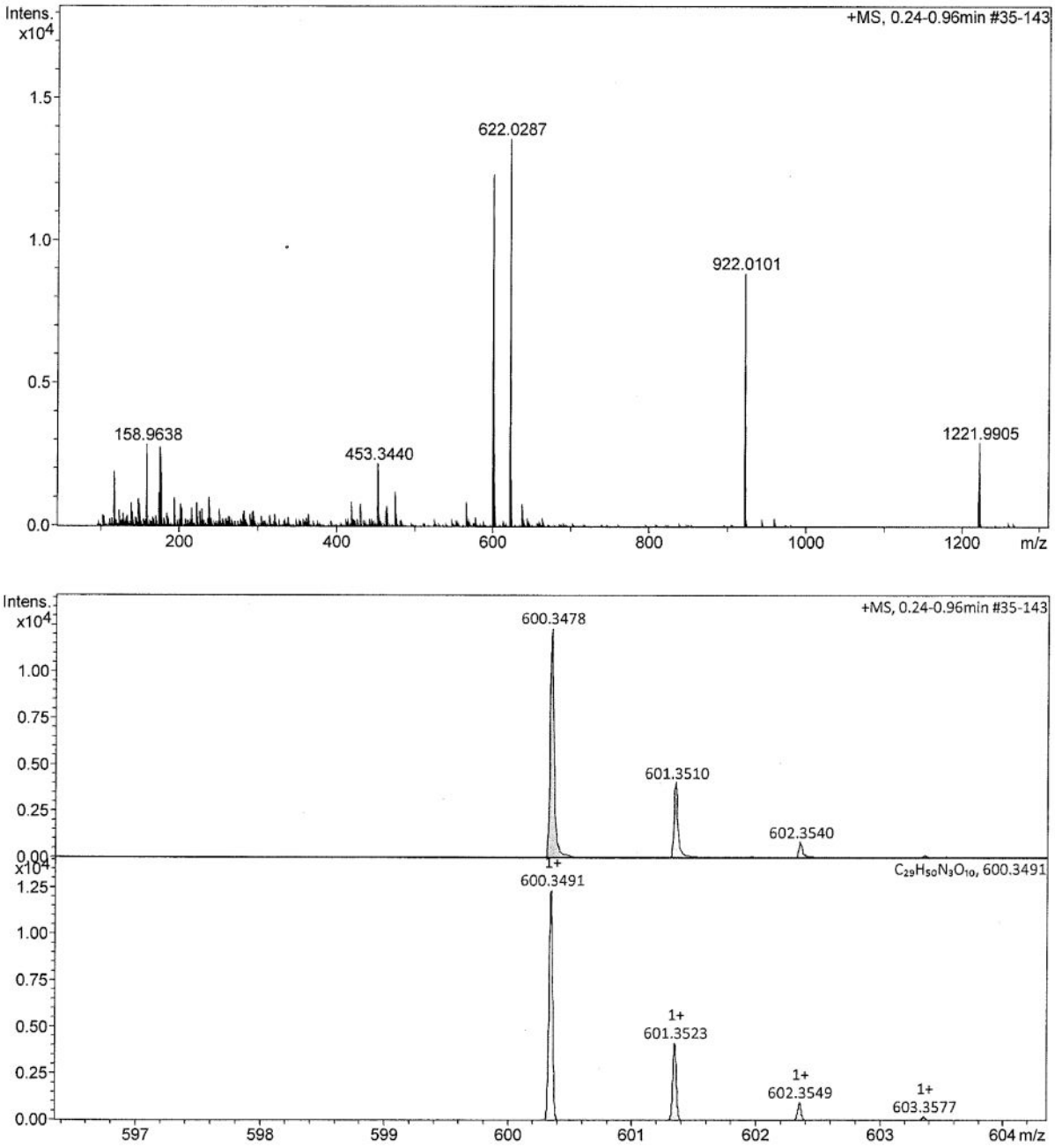




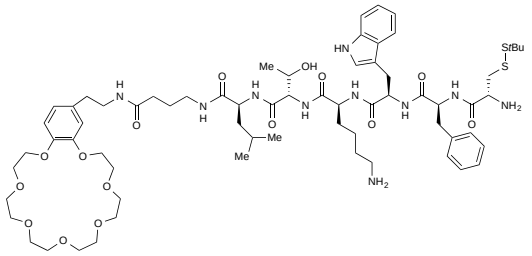
Unprotected hydroxylamine S18



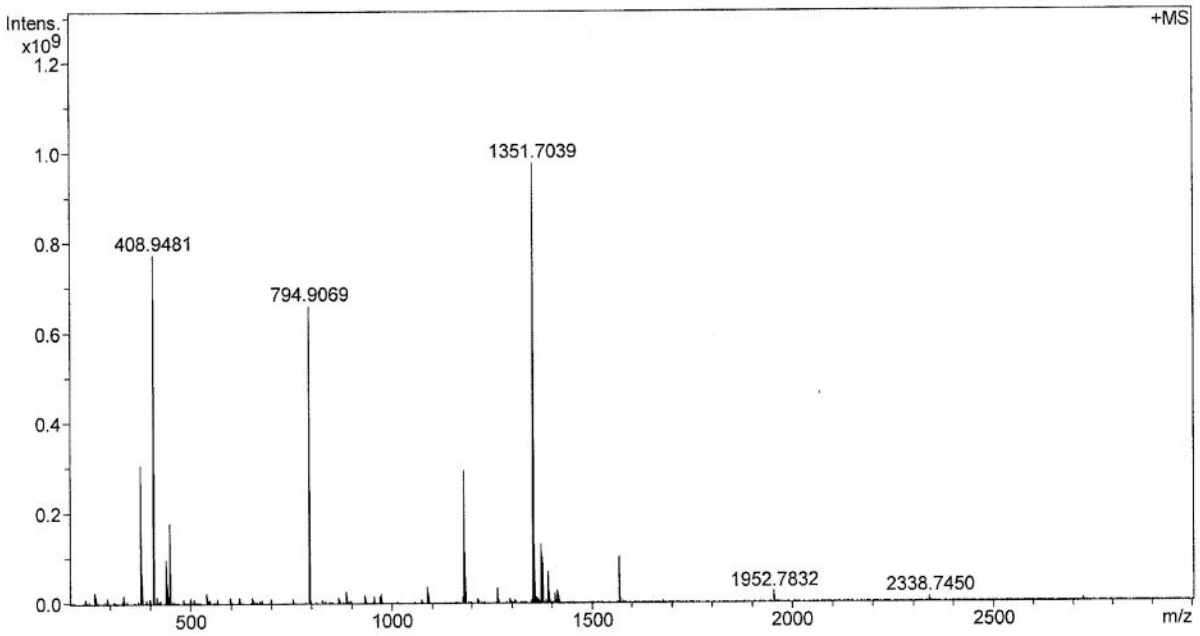
Box 1: Full scale spectra with internal reference peaks (Tunemix (pos) ESI-TOF Spezial: 118.0863, 322.0481, 622.0290, 922.0098, 1221.9906); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.

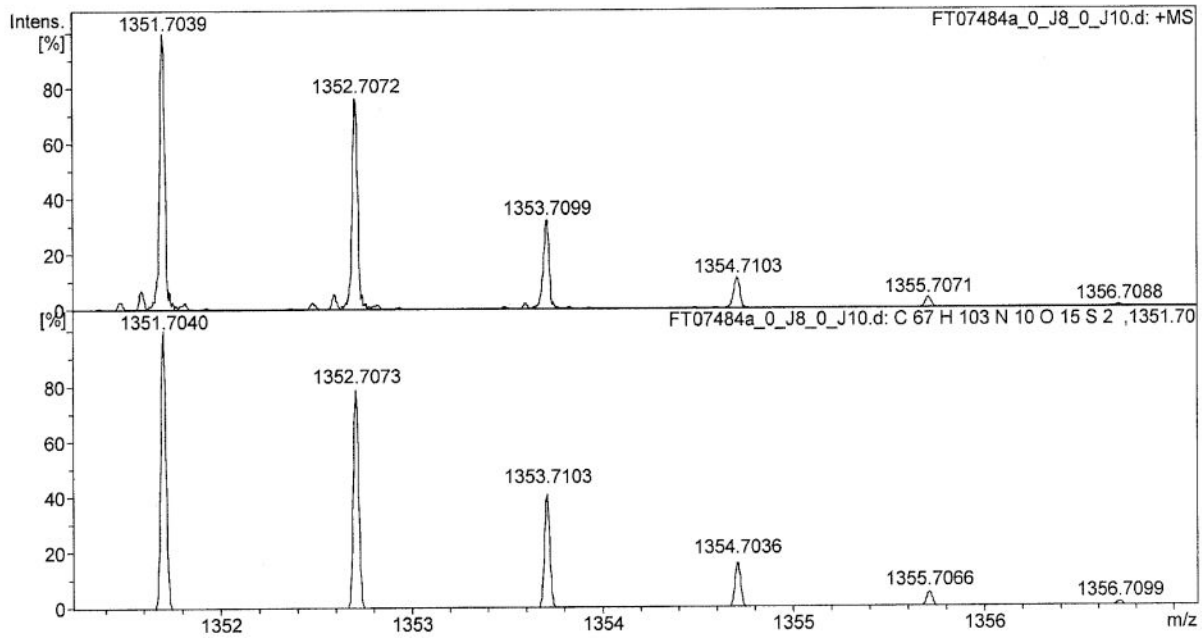


KAHA ligation product S19

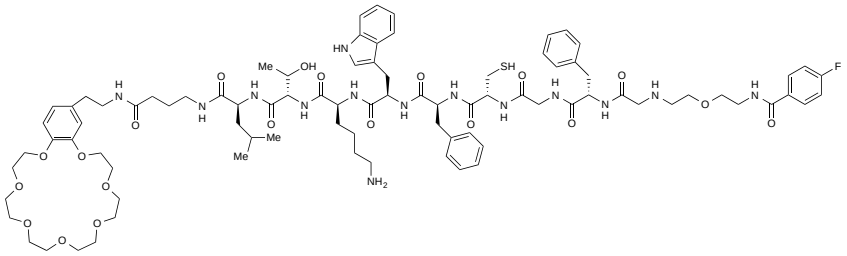


Box 1: Full scale spectra with internal reference peaks (ESI: Na-PFHA 408.9481, 794.9069, 1180.8657, 1566.8246); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.

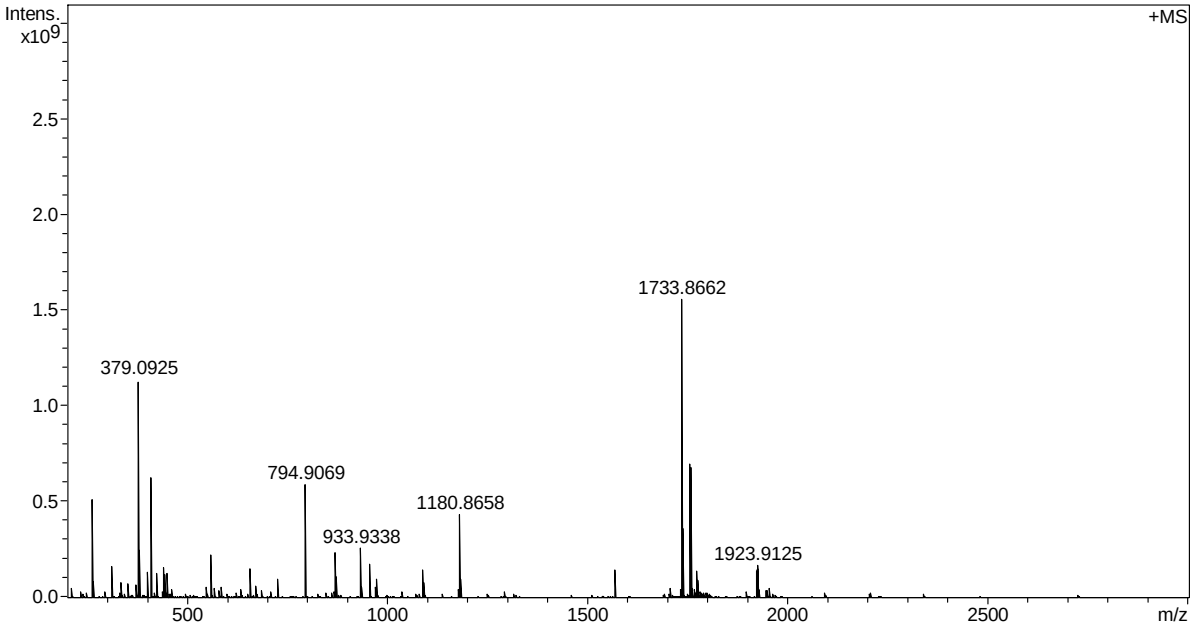


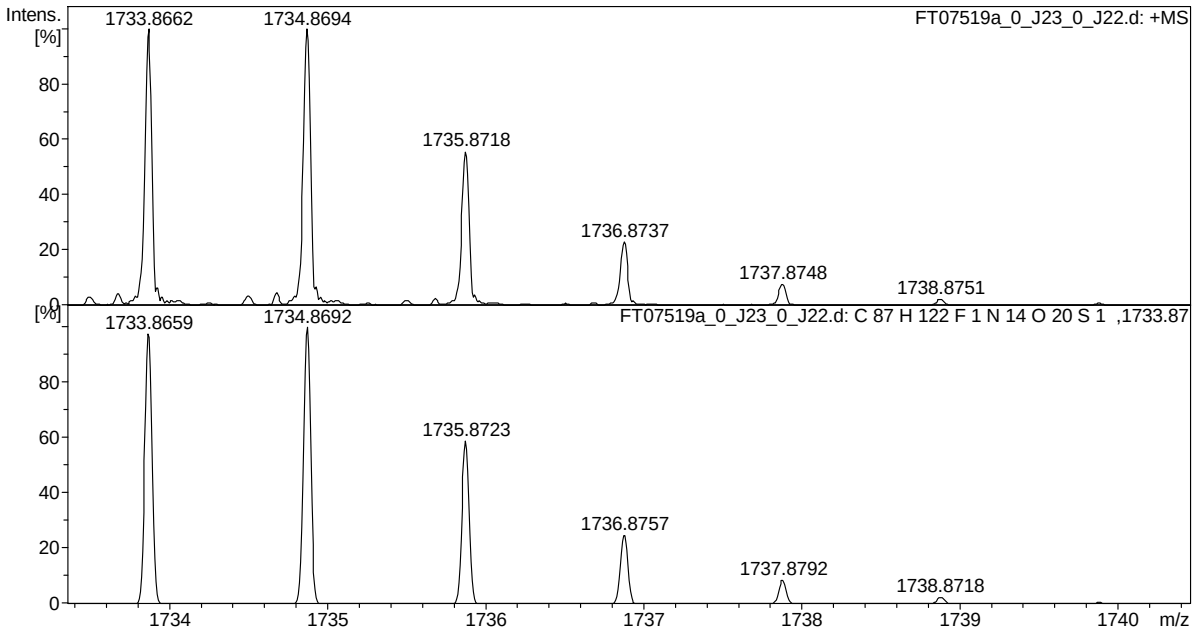


NCL product S20

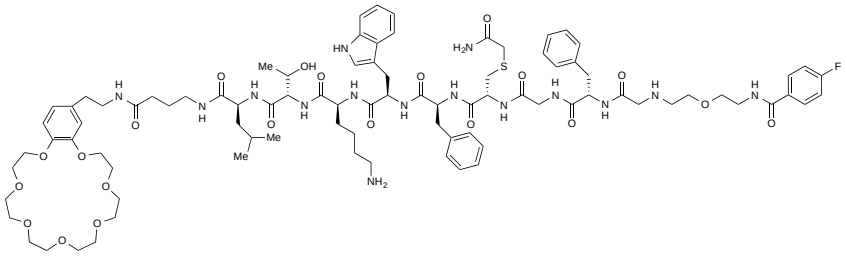


Box 1: Full scale spectra with internal reference peaks (ESI: Na-PFHA 408.9481, 794.9069, 1180.8657, 1566.8246, 1952.7834); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.

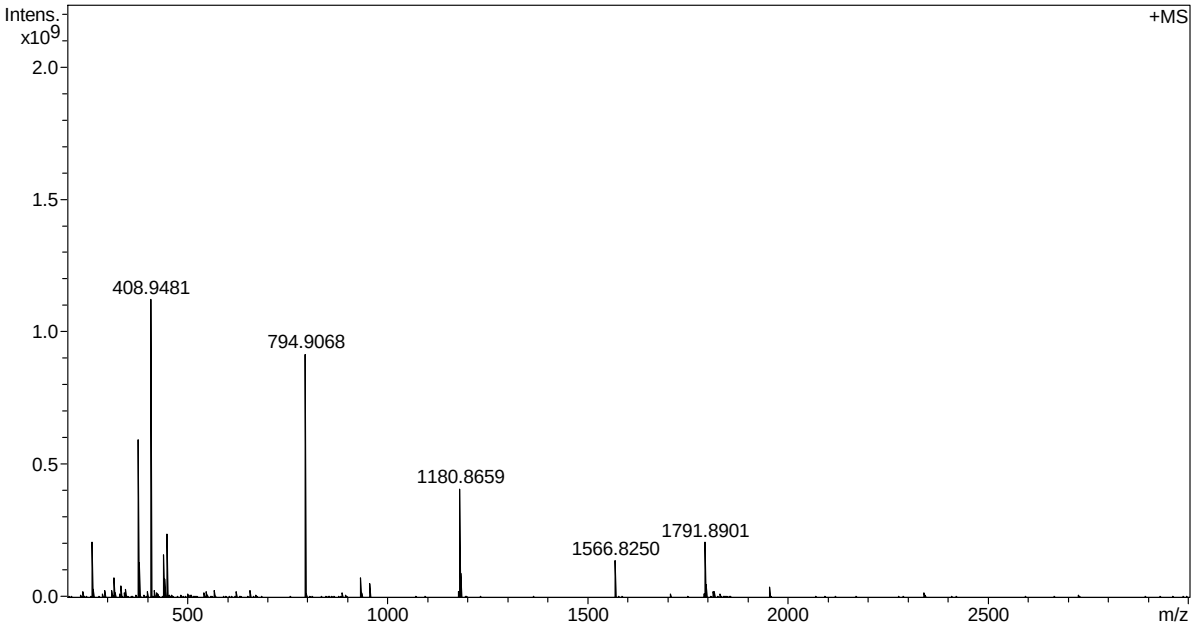


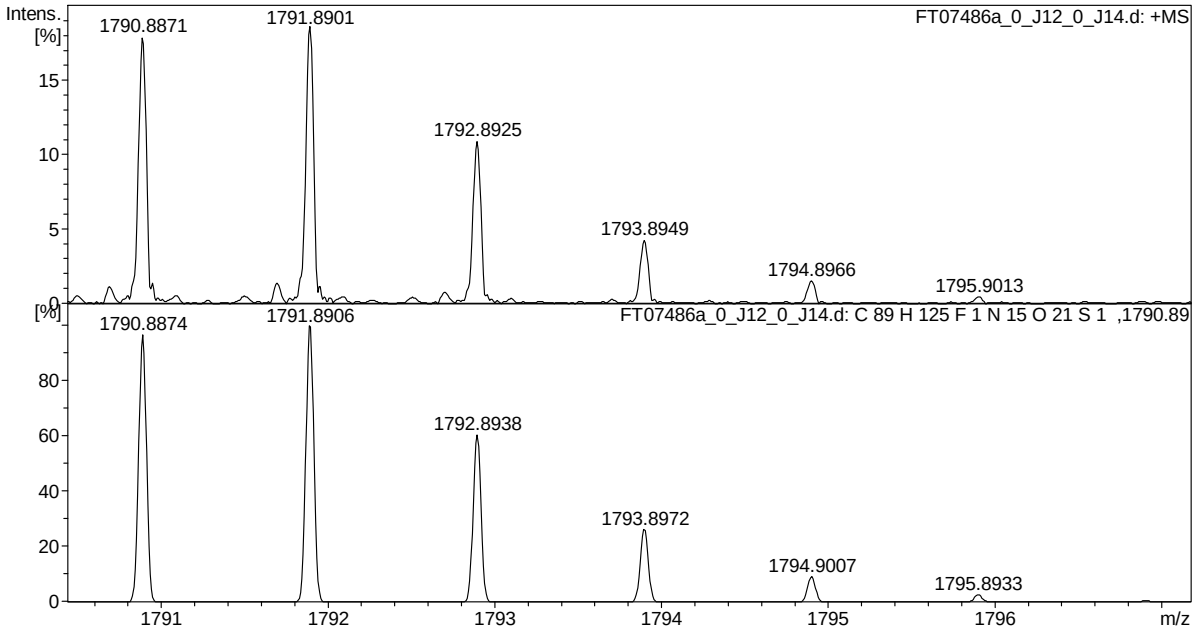


Branched-cyclic peptide B1

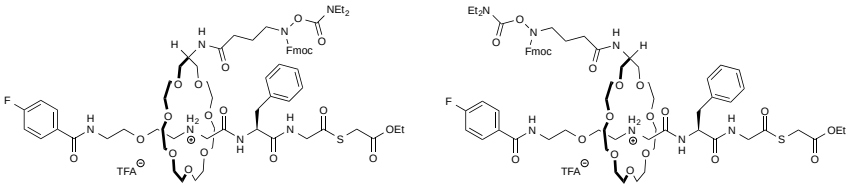


Box 1: Full scale spectra with internal reference peaks (ESI: Na-PFHA 408.9481, 794.9069, 1180.8657, 1566.8246, 1952.7834); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.

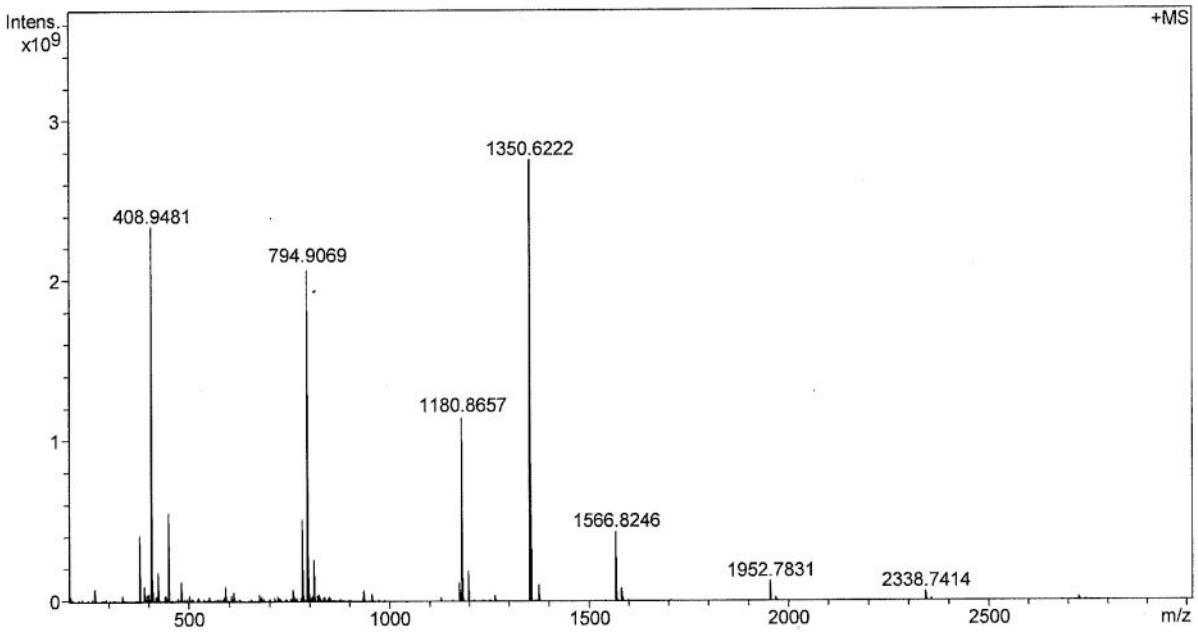


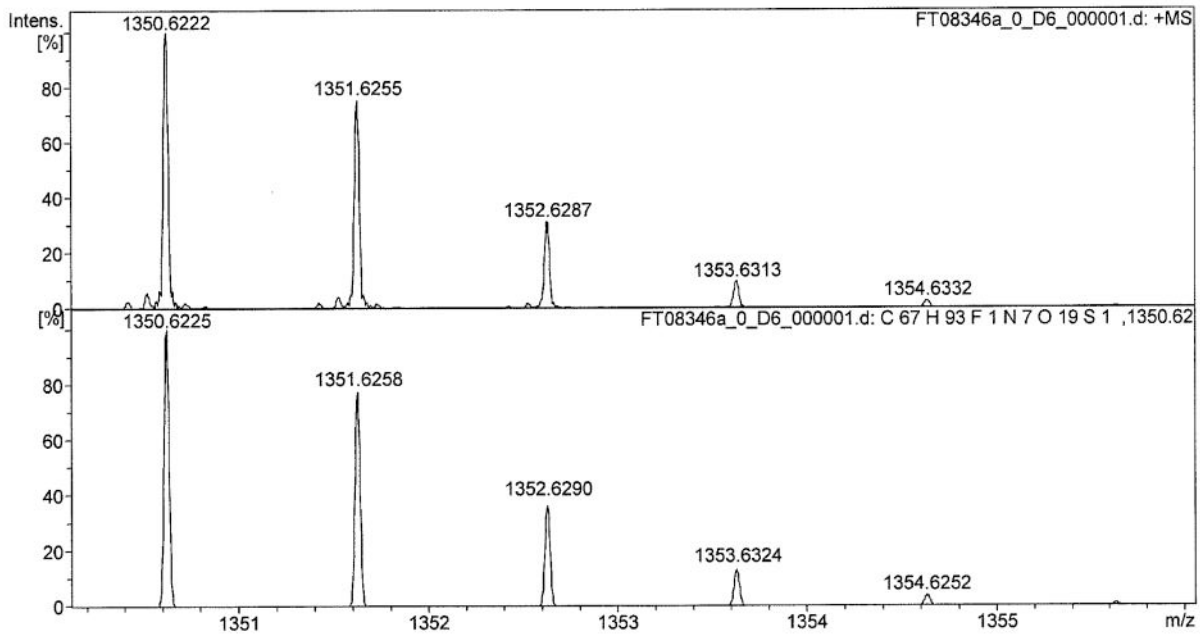


[2]Rotaxane S25

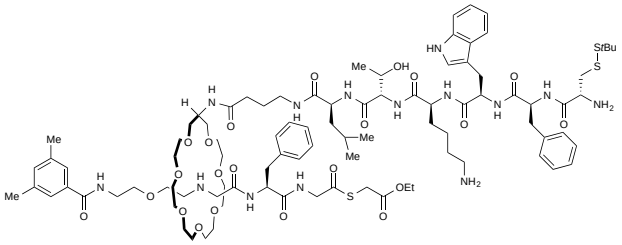


Box 1: Full scale spectra with internal reference peaks (ESI: Na-PFHA 408.9481, 794.9069, 1180.8657, 1566.8246); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.

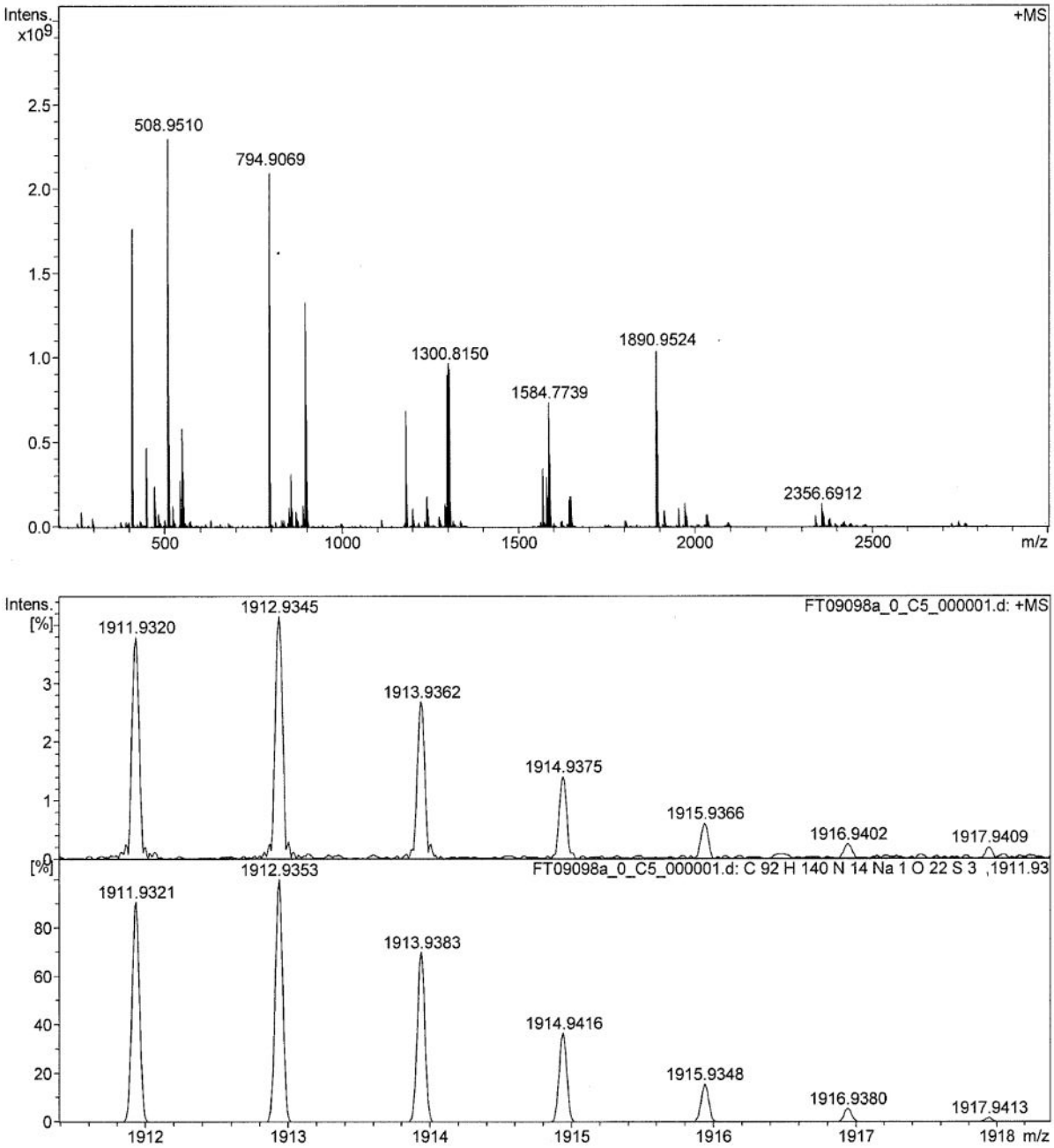




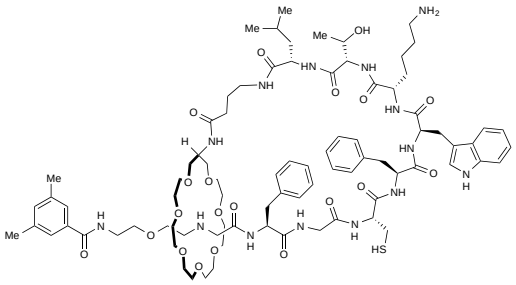
Peptido[2]rotaxane S26



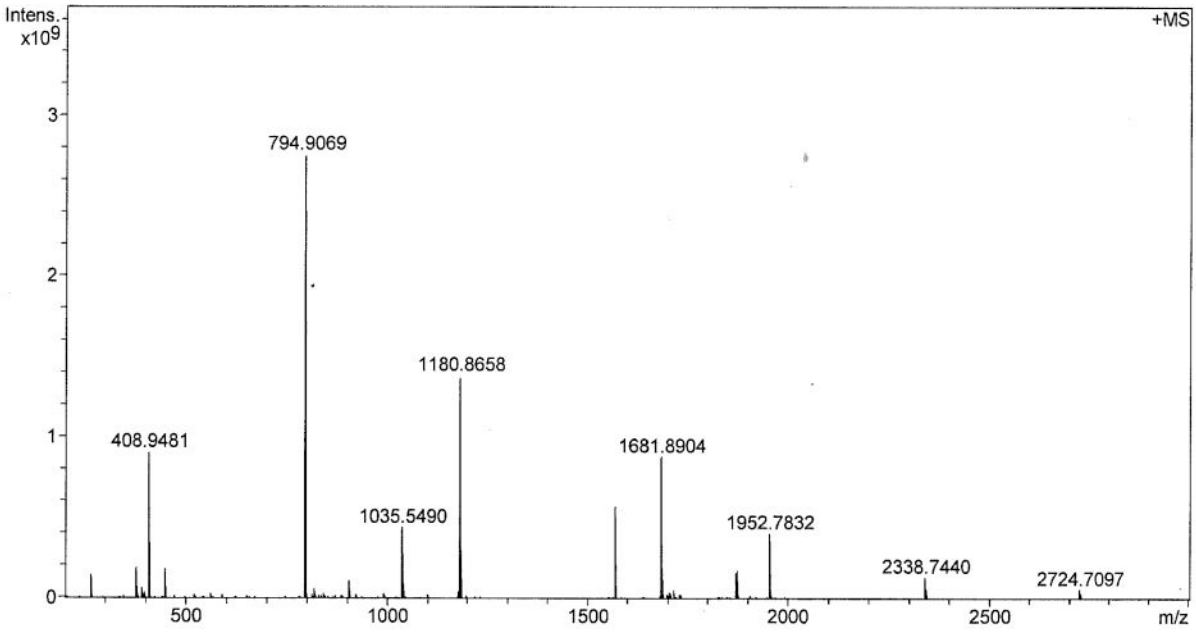
Box 1: Full scale spectra with internal reference peaks (ESI: Na-PFHA 794.9069, 1180.8657, 1566.8246, 1952.7834, 2338.7423, 2724.7011); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.

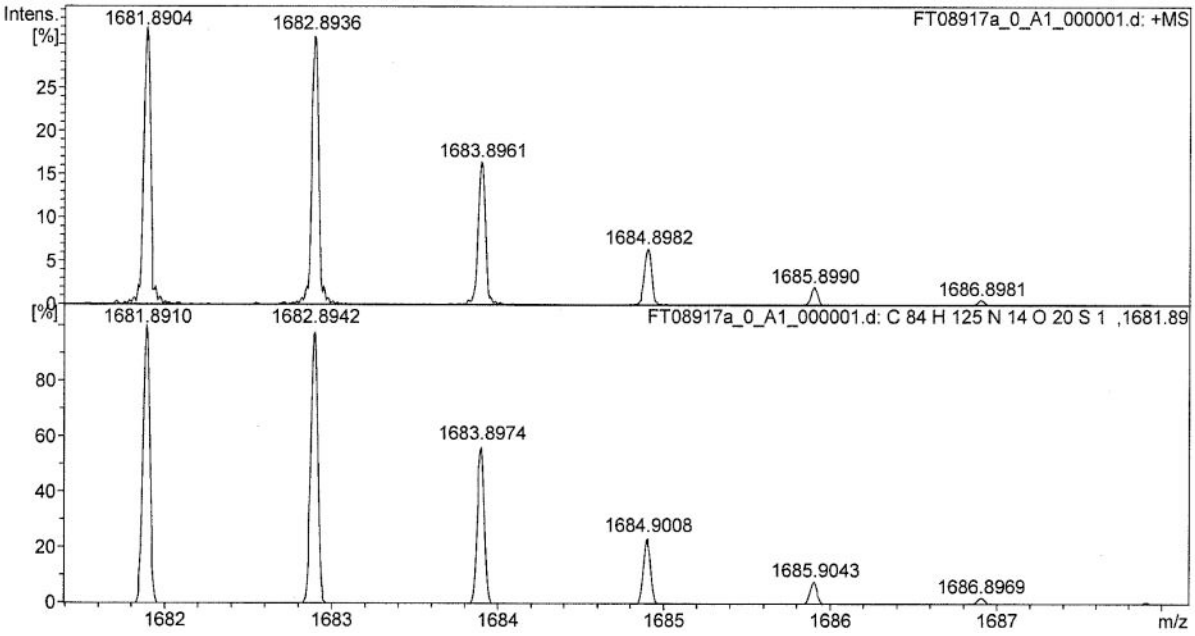


Lasso peptide S27

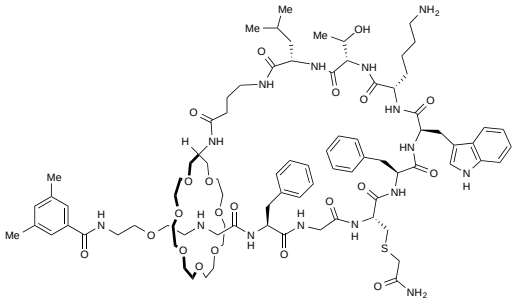


Box 1: Full scale spectra with internal reference peaks (ESI: Na-PFHA 408.9481, 794.9069, 1180.8657, 1566.8246, 1952.7834); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.

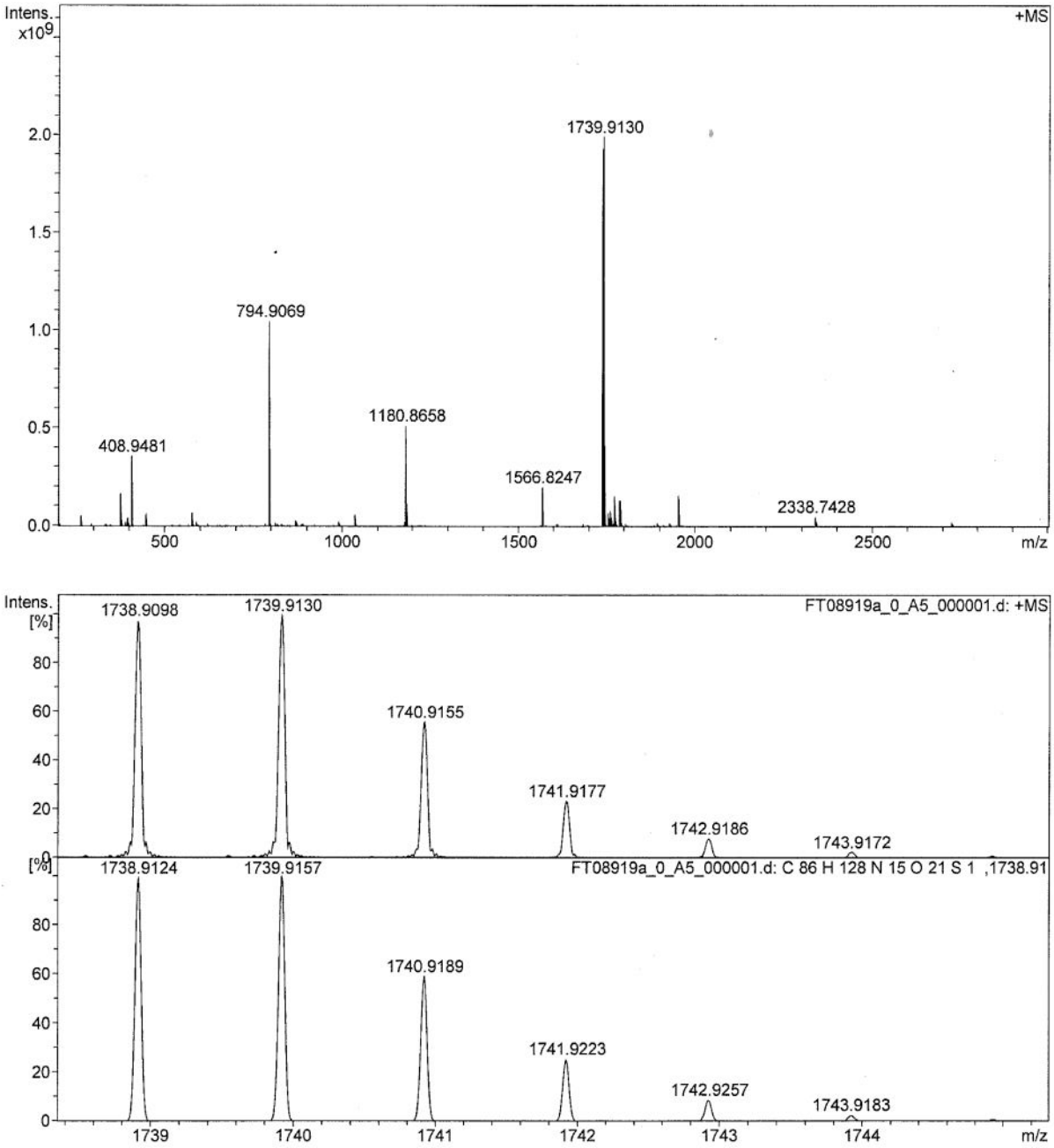




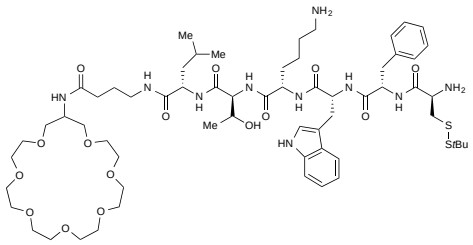
Cys-alkylated lasso peptide L2



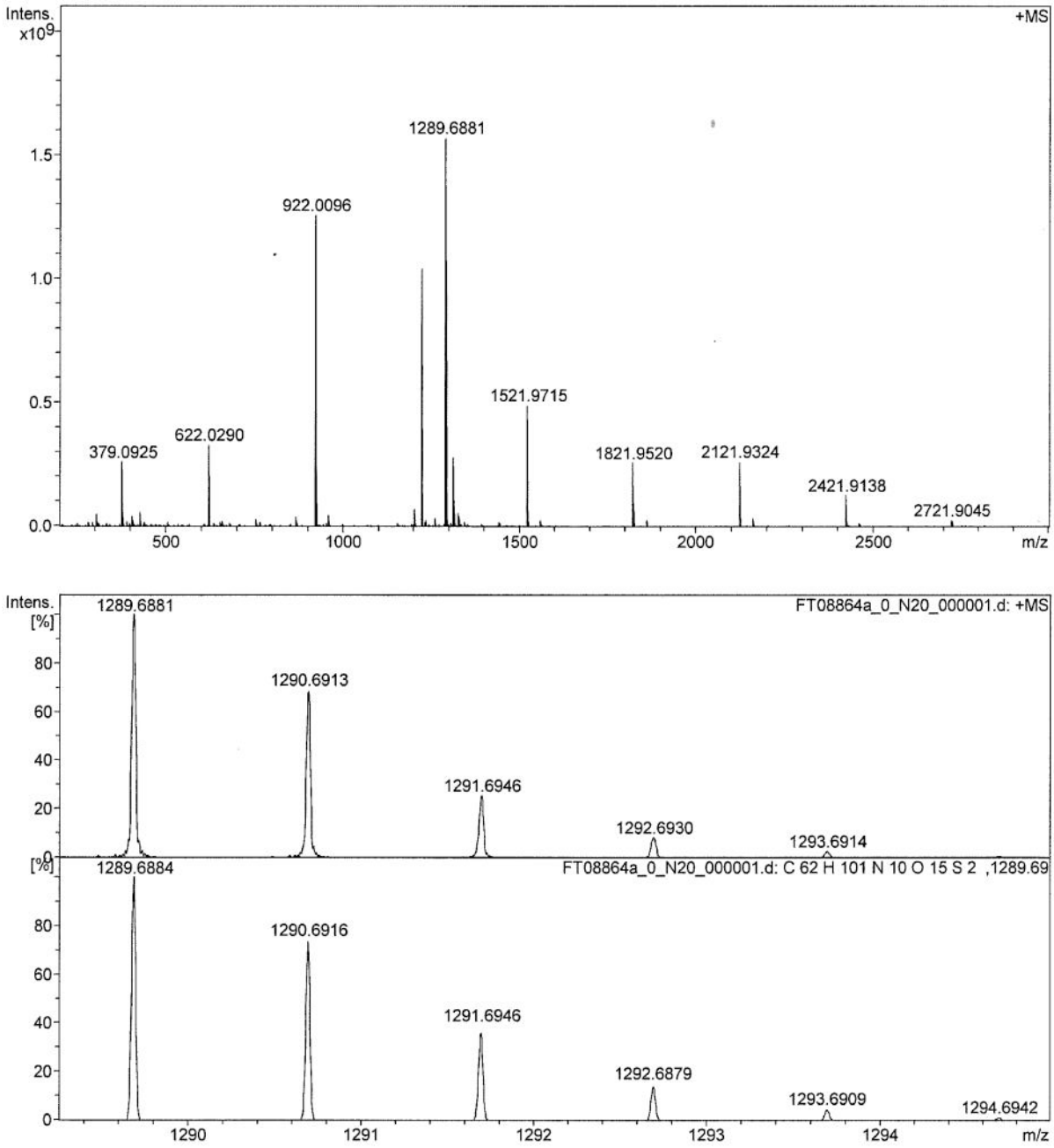
Box 1: Full scale spectra with internal reference peaks (ESI: Na-PFHA 408.9481, 794.9069, 1180.8657, 1566.8246, 1952.7834, 2338.7423); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.



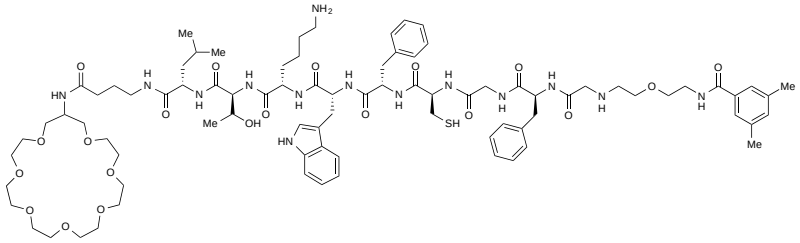
KAHA ligation product S28



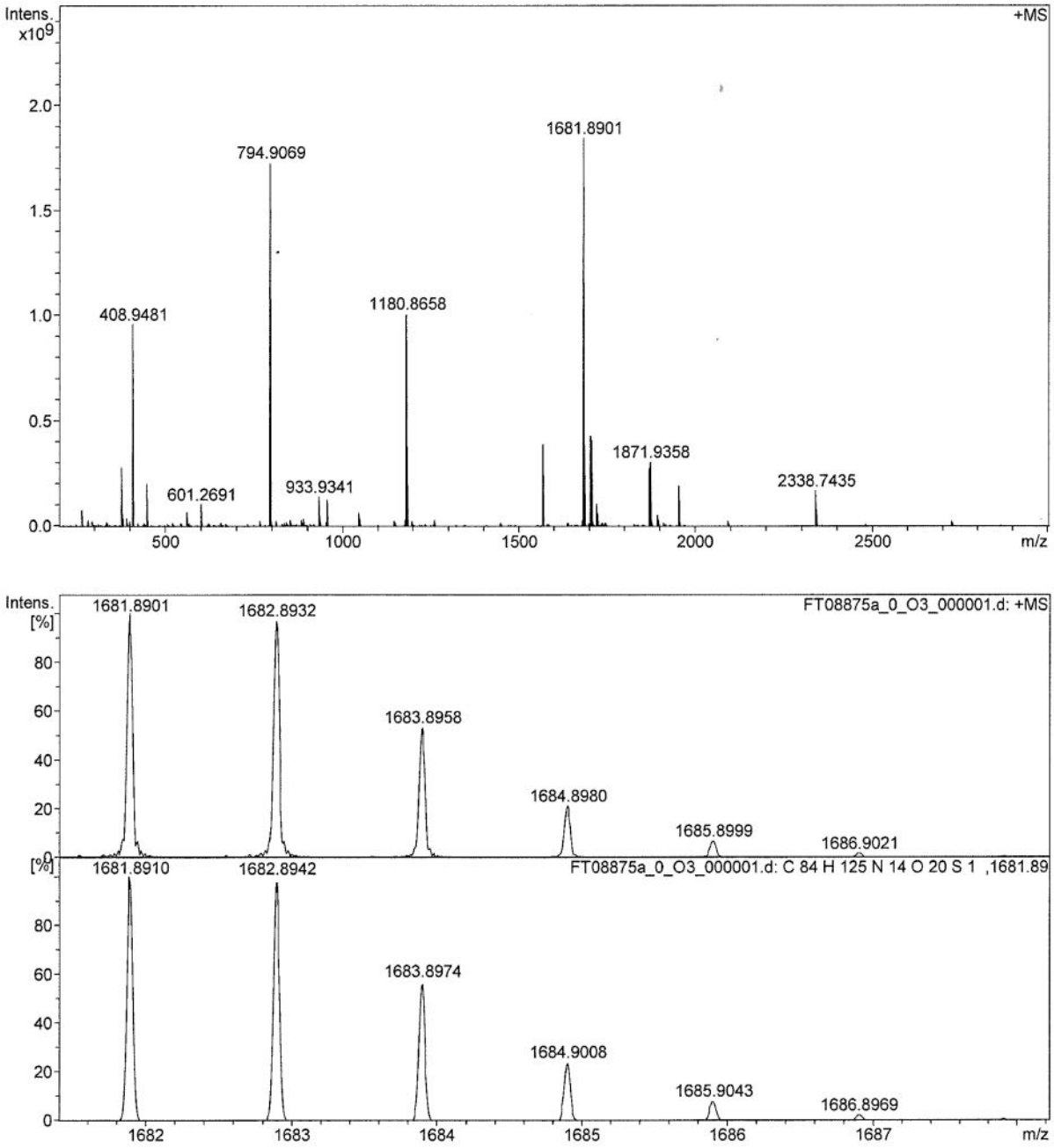
Box 1: Full scale spectra with internal reference peaks (ESI: Tuning Mix ES-TOF (ESI) (pos)DCTB 322.0481, 622.0290, 1221.9906, 1521.9715, 2421.9140); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.



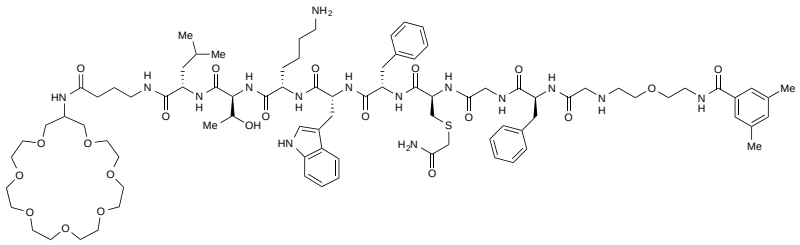
NCL product S29



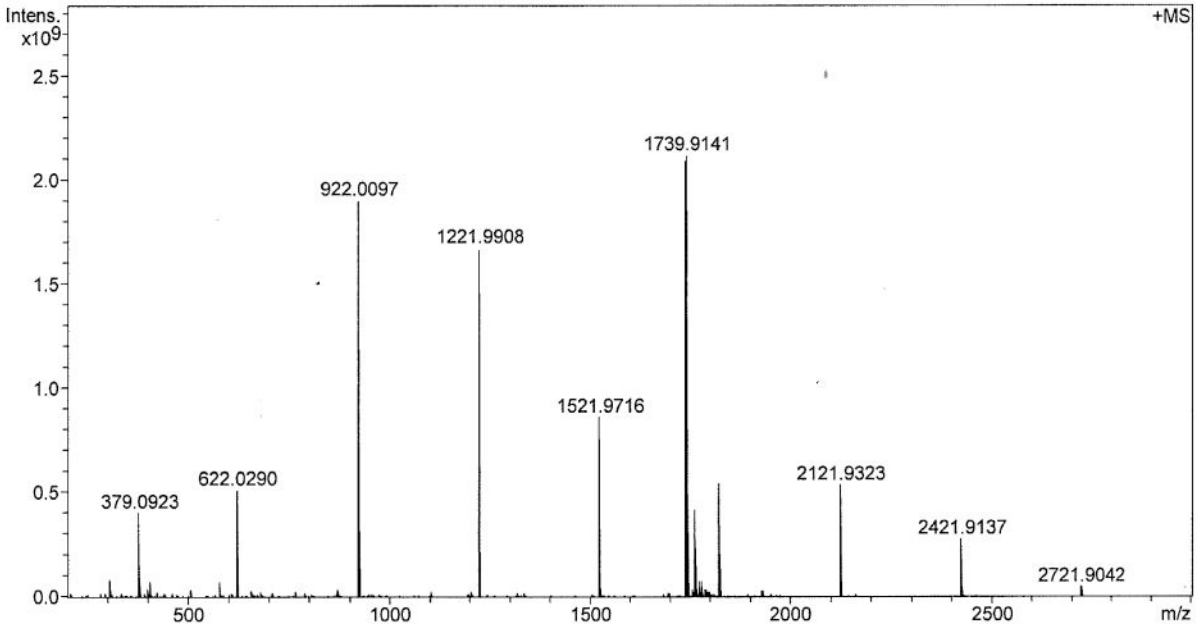
Box 1: Full scale spectra with internal reference peaks (ESI: Na-PFHA 408.9481, 794.9069, 1180.8657, 1566.8246, 1952.7834); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.

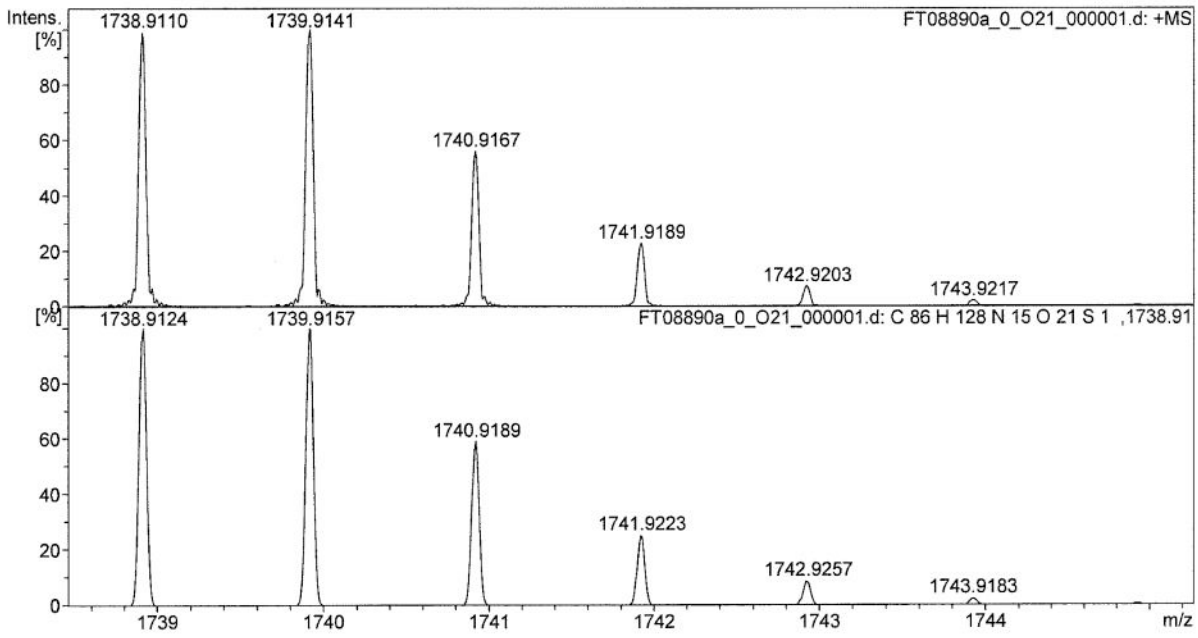


Branched-cyclic peptide B2

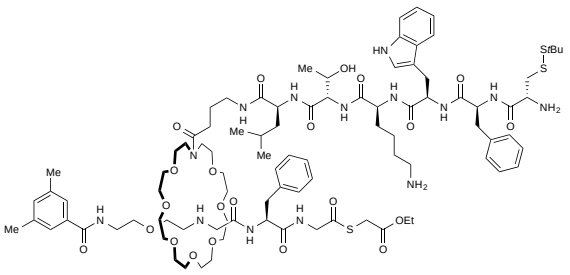


Box 1: Full scale spectra with internal reference peaks (ESI: Tuning Mix ES-TOF (ESI) (pos)DCTB 622.0290, 922.0098, 1221.9906, 1521.9715, 2421.9140); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.

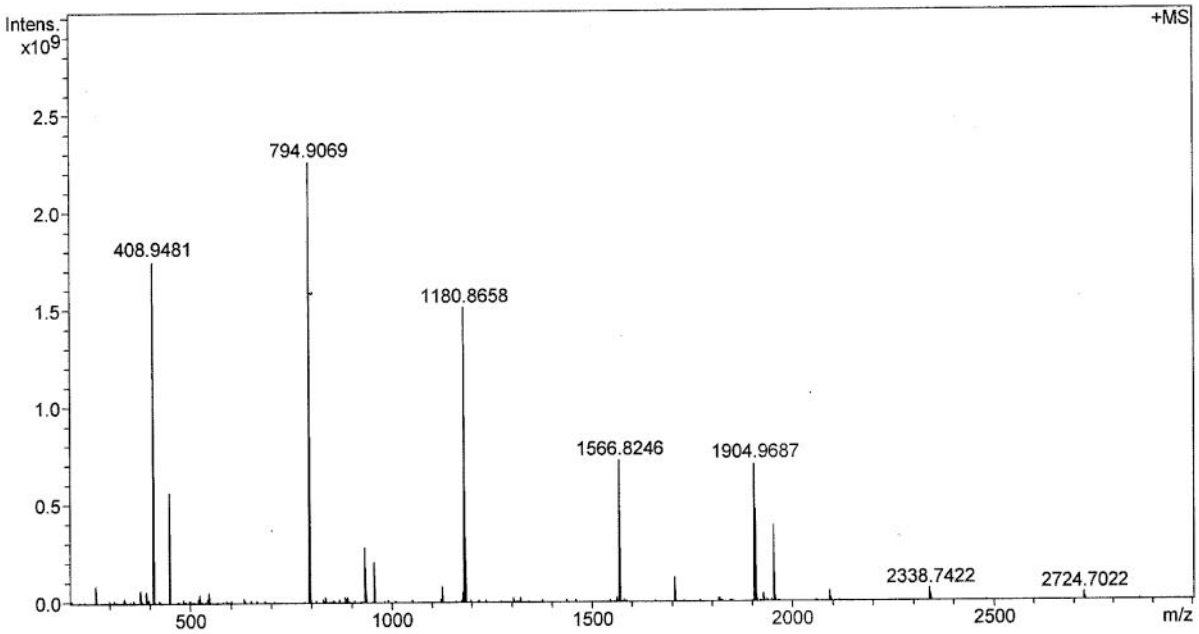


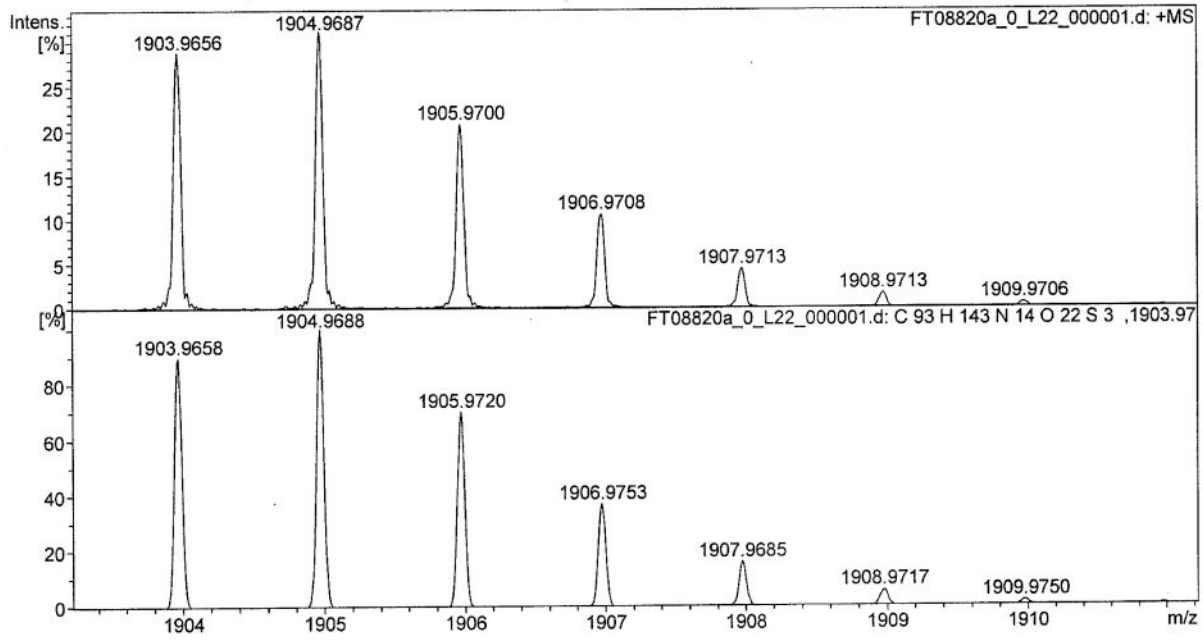


Peptido[2]rotaxane S31

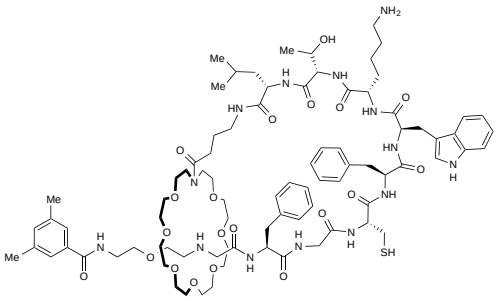


Box 1: Full scale spectra with internal reference peaks (ESI: Na-PFHA 408.9481, 794.9069, 1180.8657, 1566.8246, 2338.7423); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.

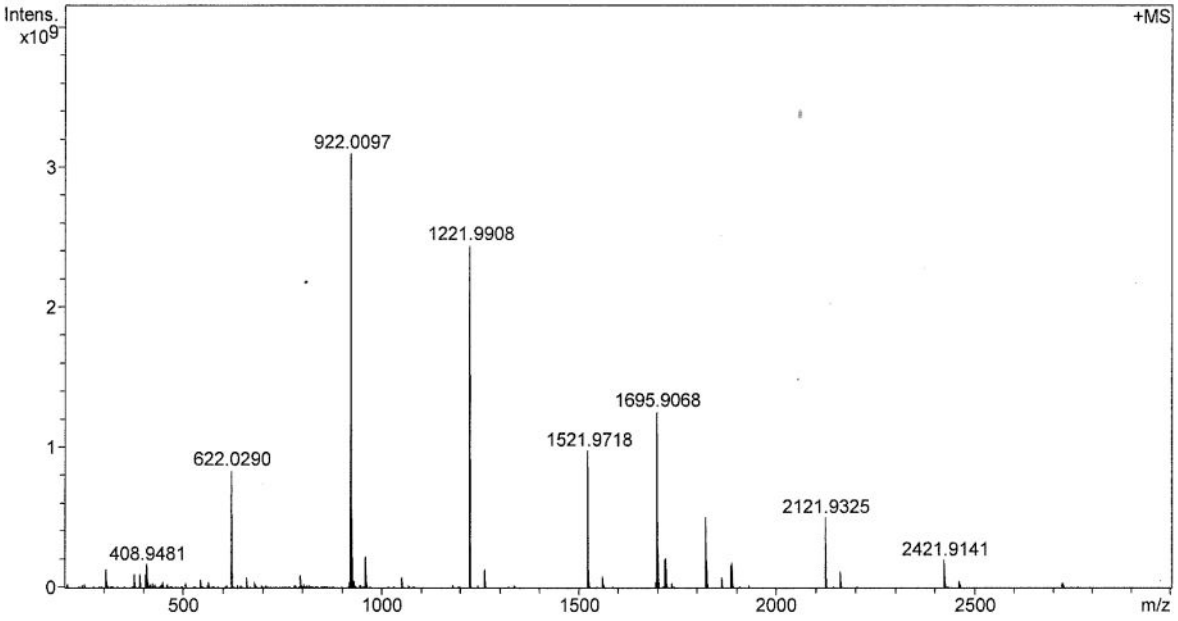


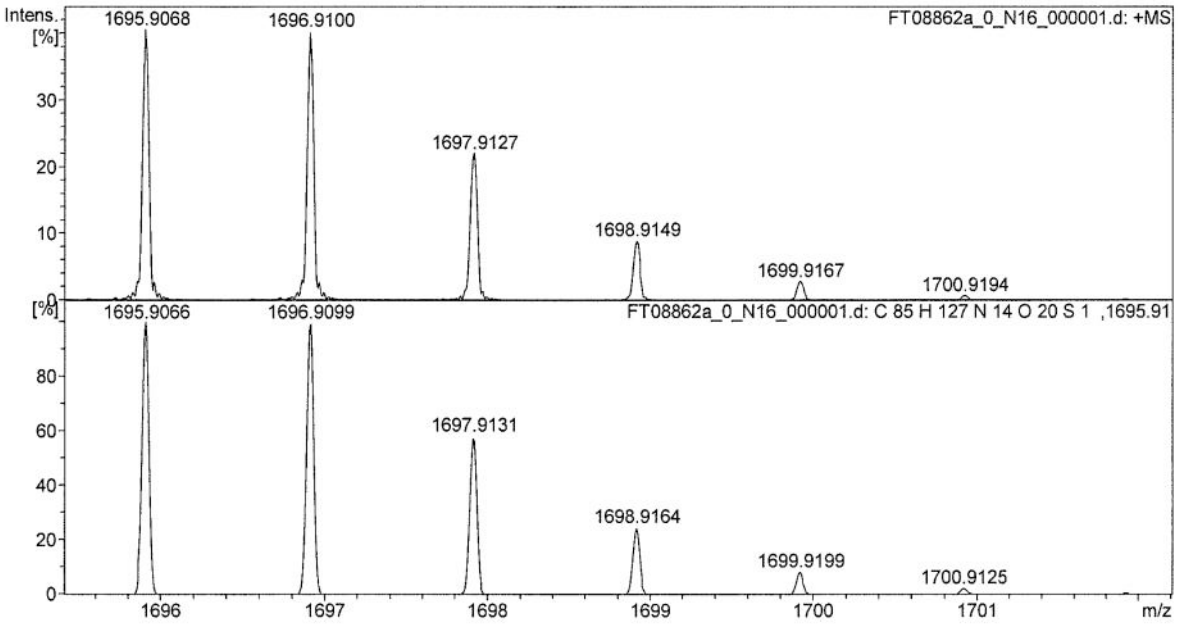


Lasso peptide S32

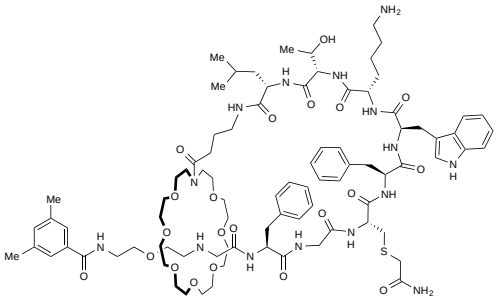


Box 1: Full scale spectra with internal reference peaks (ESI: Tuning Mix ES-TOF (ESI) (pos)DCTB 322.0481, 622.0290, 922.0098, 1221.9906, 1821.9523, 2421.9140); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.

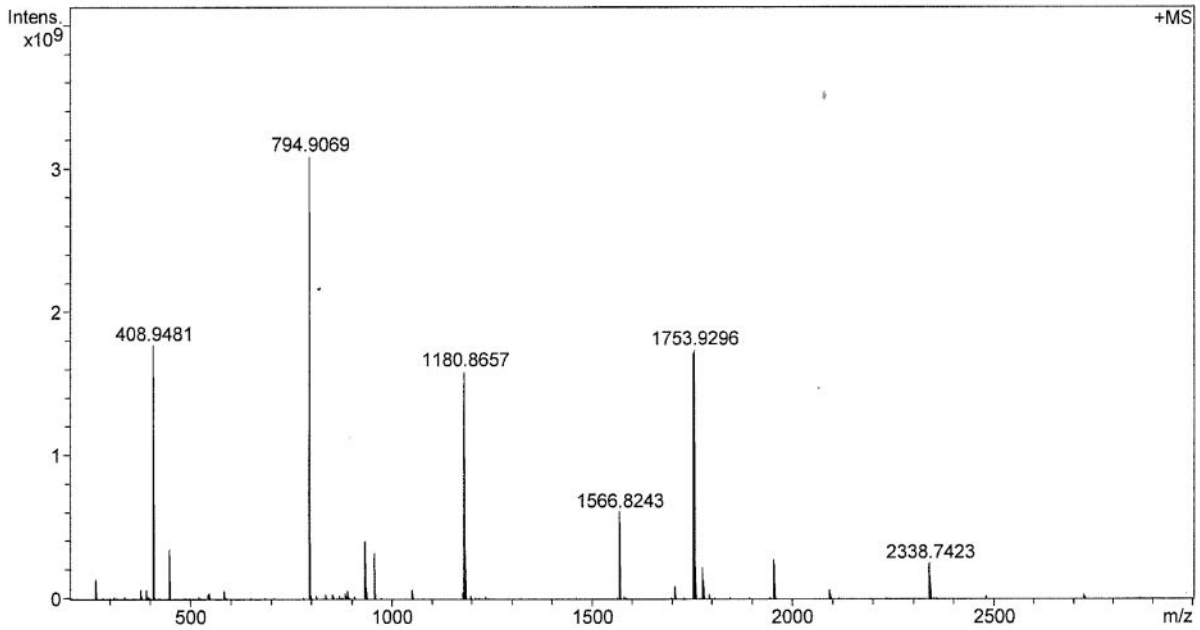


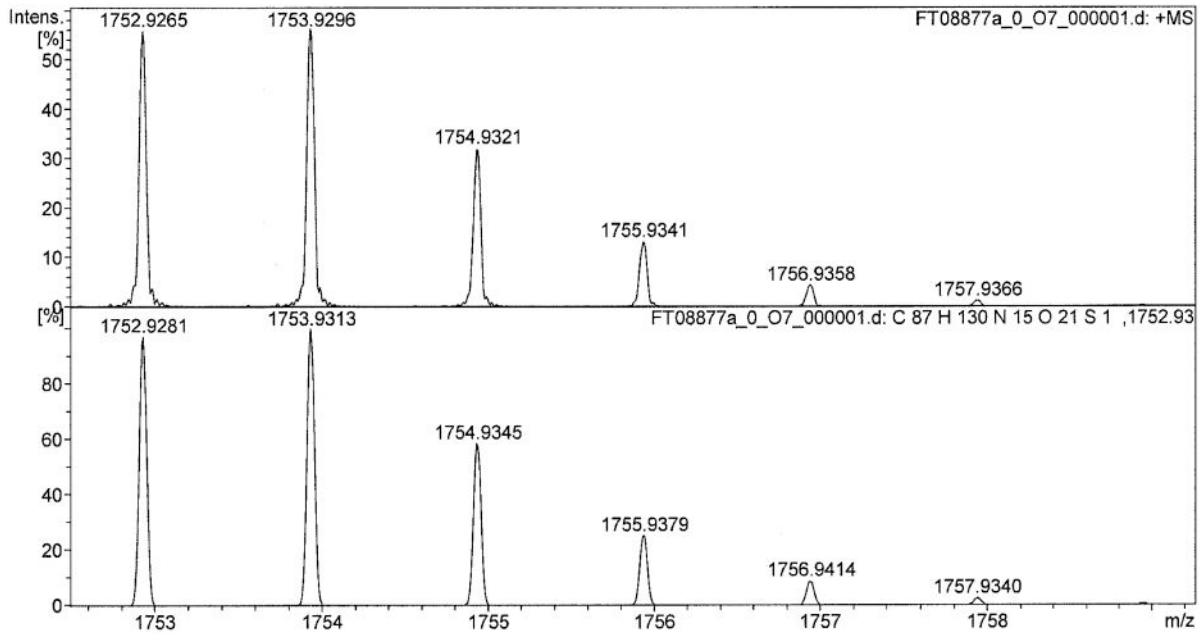


Cys-alkylated lasso peptide L3

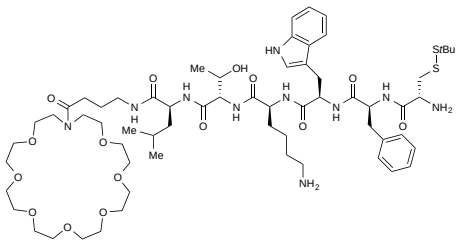


Box 1: Full scale spectra with internal reference peaks (ESI: Na-PFHA 408.9481, 794.9069, 1180.8657, 1952.7834, 2338.7423); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.

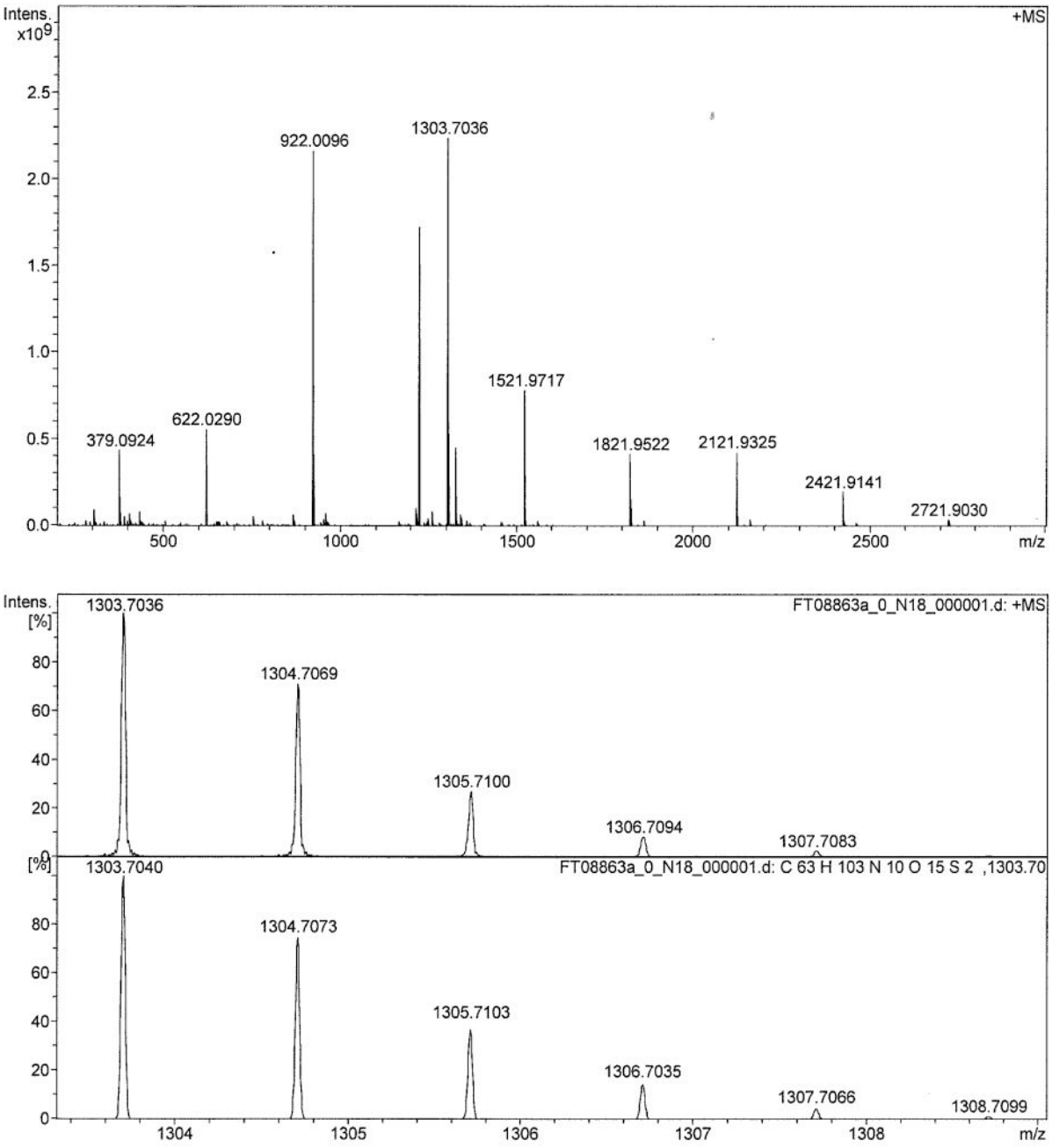




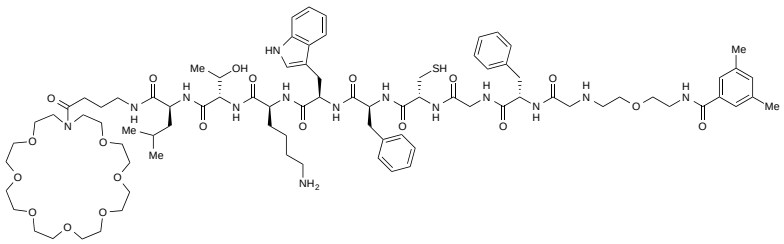
KAHA ligation product S33



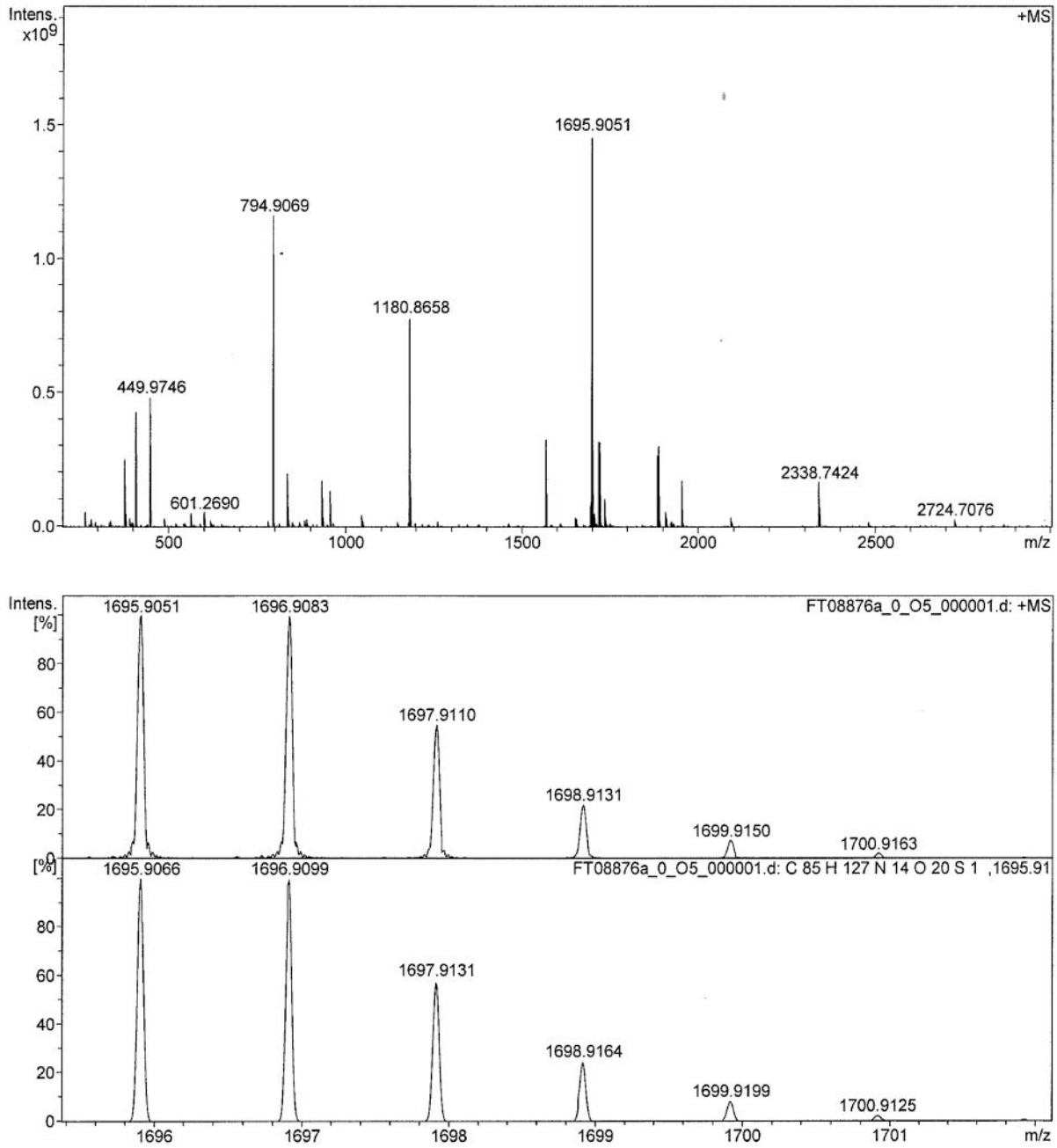
Box 1: Full scale spectra with internal reference peaks (ESI: Tuning Mix ES-TOF (ESI) (pos)DCTB 322.0481, 622.0290, 1221.9906, 1821.9523, 2421.9140); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.



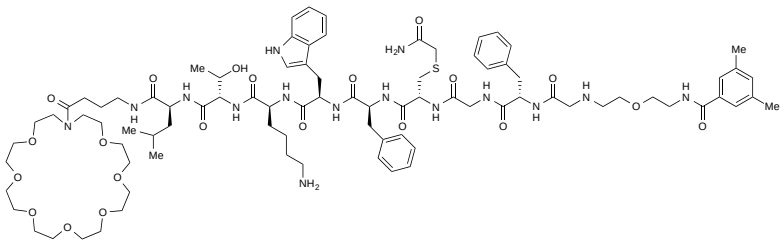
NCL product S34



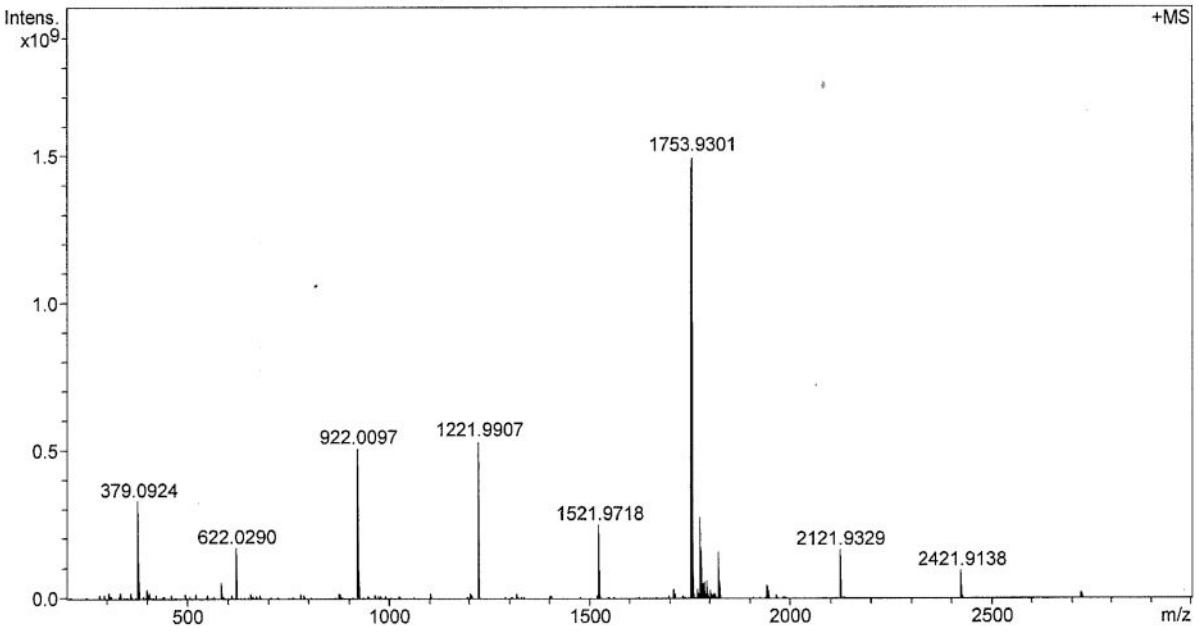
Box 1: Full scale spectra with internal reference peaks (ESI: Na-PFHA 408.9481, 794.9069, 1180.8657, 1566.8246, 1952.7834, 2338.7423); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.

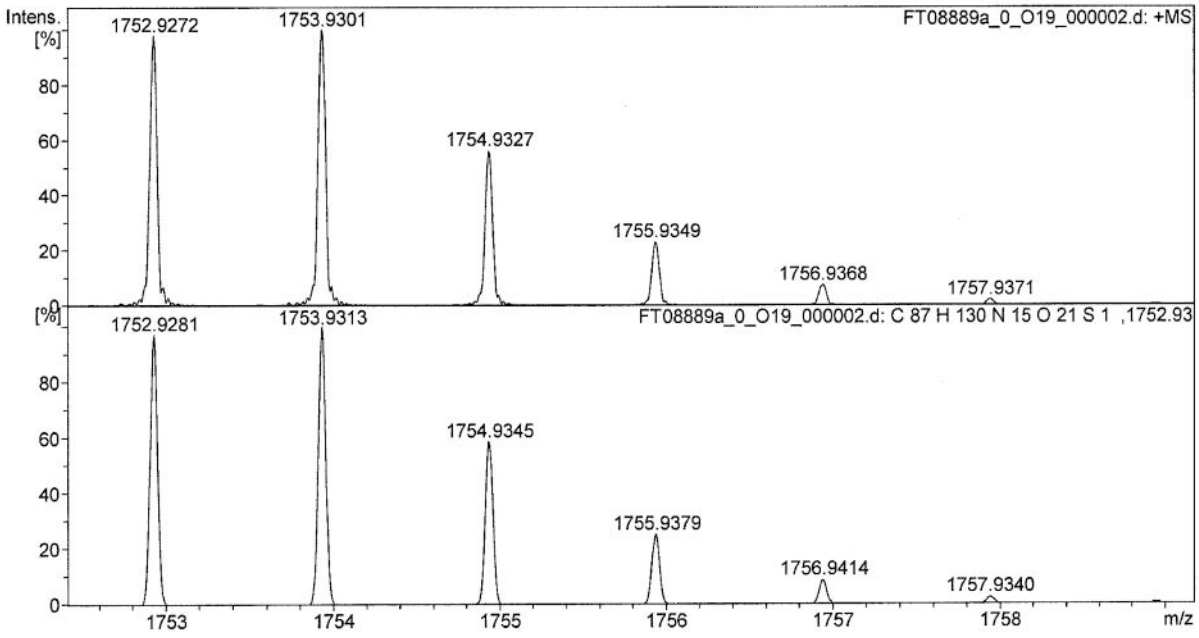


Branched-cyclic peptide B3

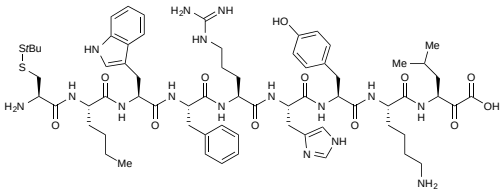


Box 1: Full scale spectra with internal reference peaks (ESI: Tuning Mix ES-TOF (ESI) (pos)DCTB 622.0290, 922.0098, 1221.9906, 1521.9715, 2121.9332, 2421.9140); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.

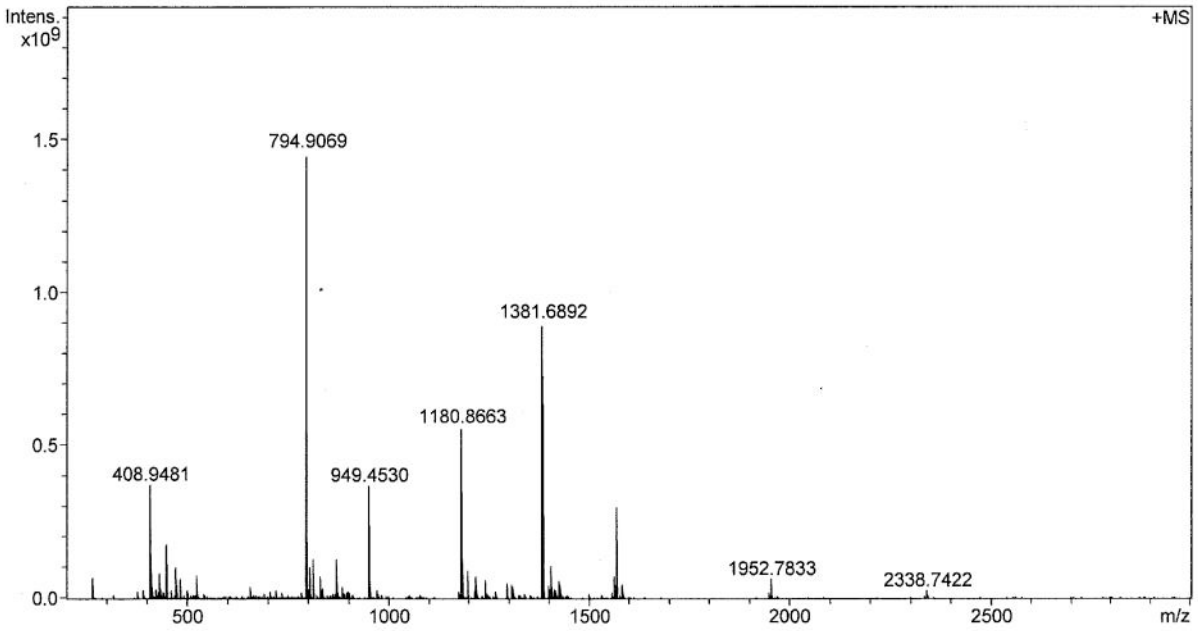


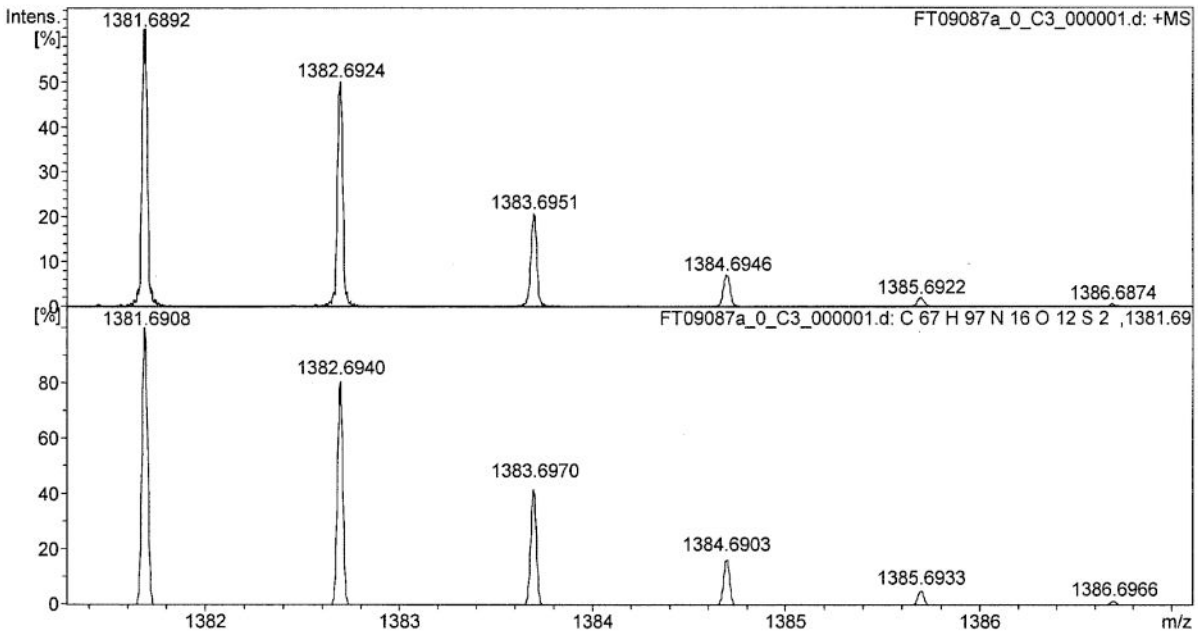


Peptide α-ketoacid Fc binder S35

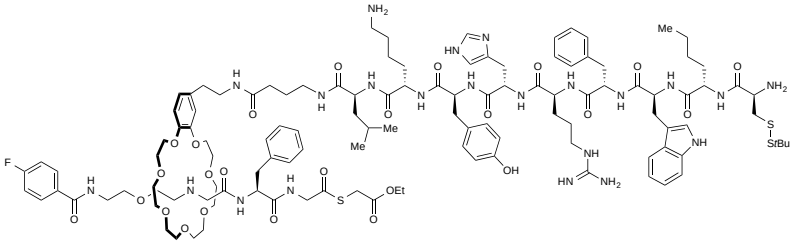


Box 1: Full scale spectra with internal reference peaks (ESI: Na-PFHA 408.9481, 794.9069, 1566.8246, 1952.7834, 2338.7423); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.

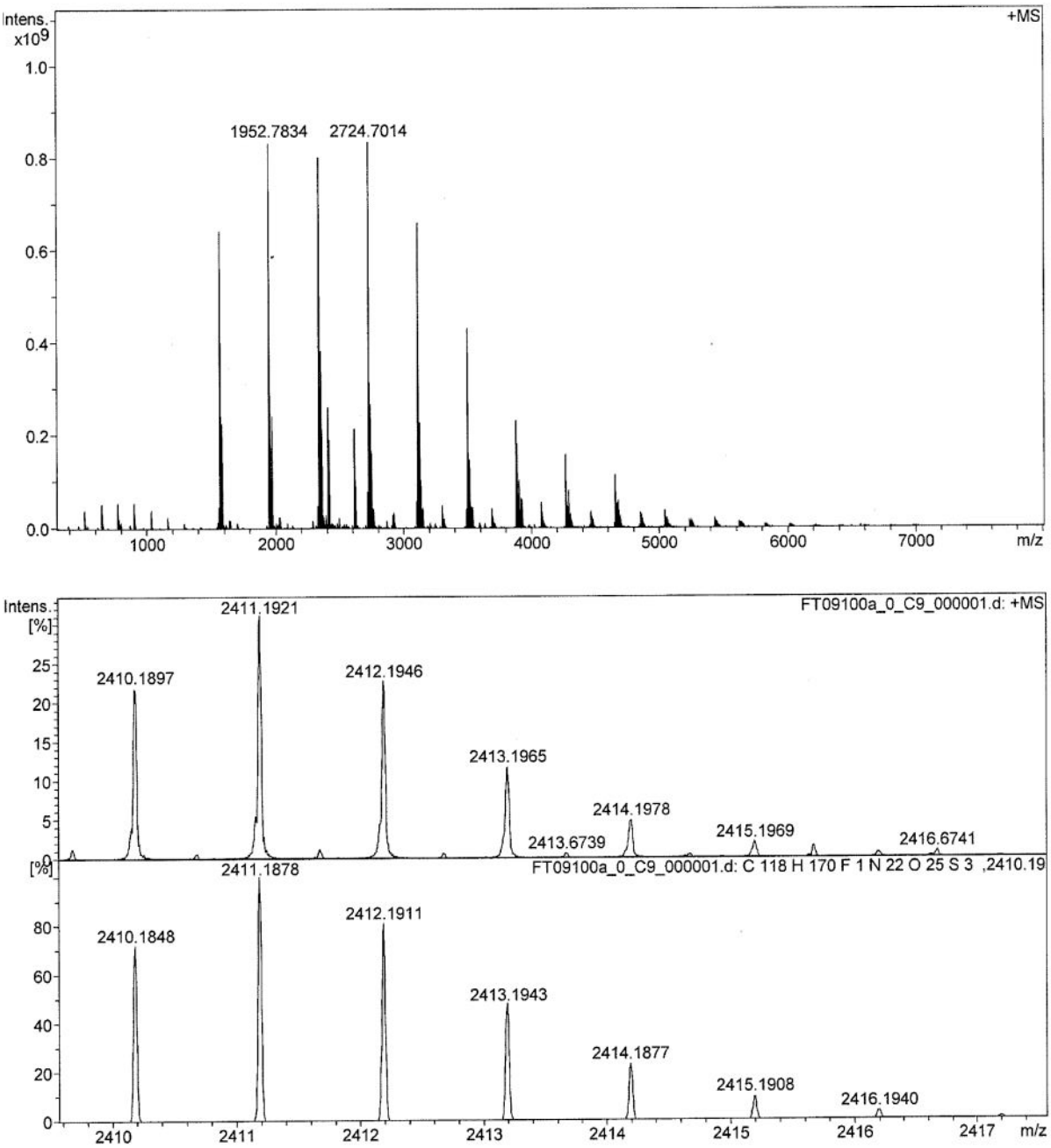




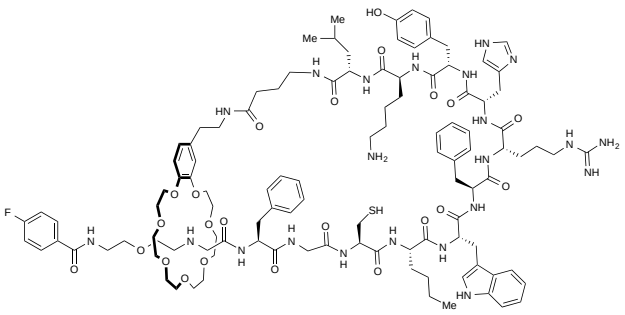
Peptido[2]rotaxane S36



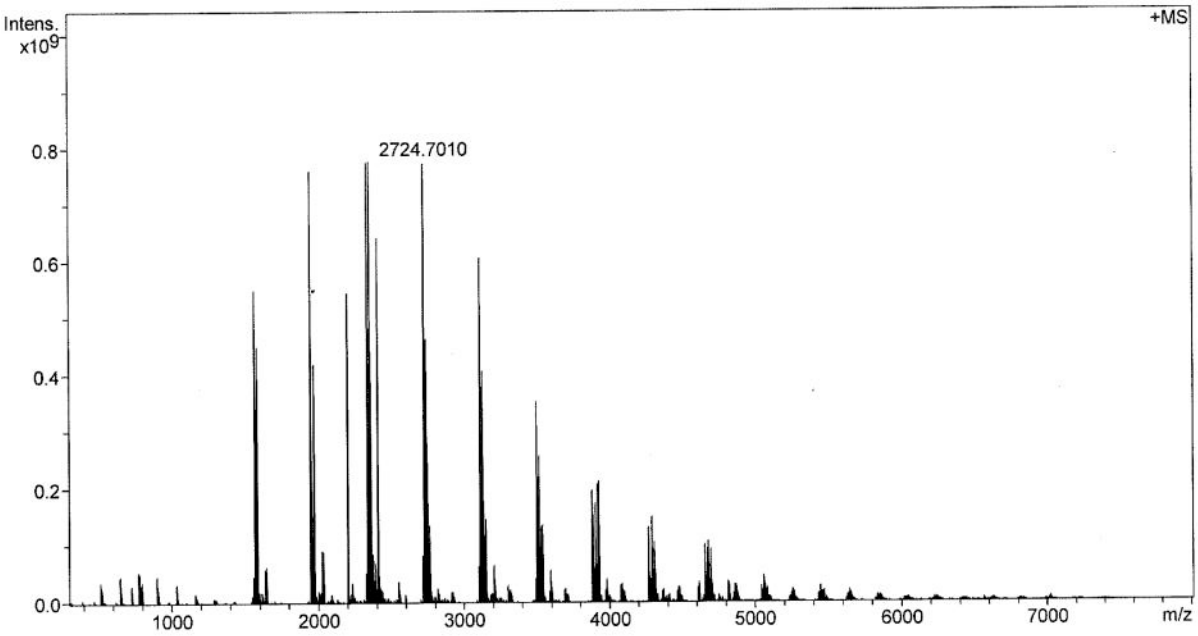
Box 1: Full scale spectra with internal reference peaks (ESI: Na-PFHA 1566.8246, 1952.7834, 2724.7011, 3110.6599); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.

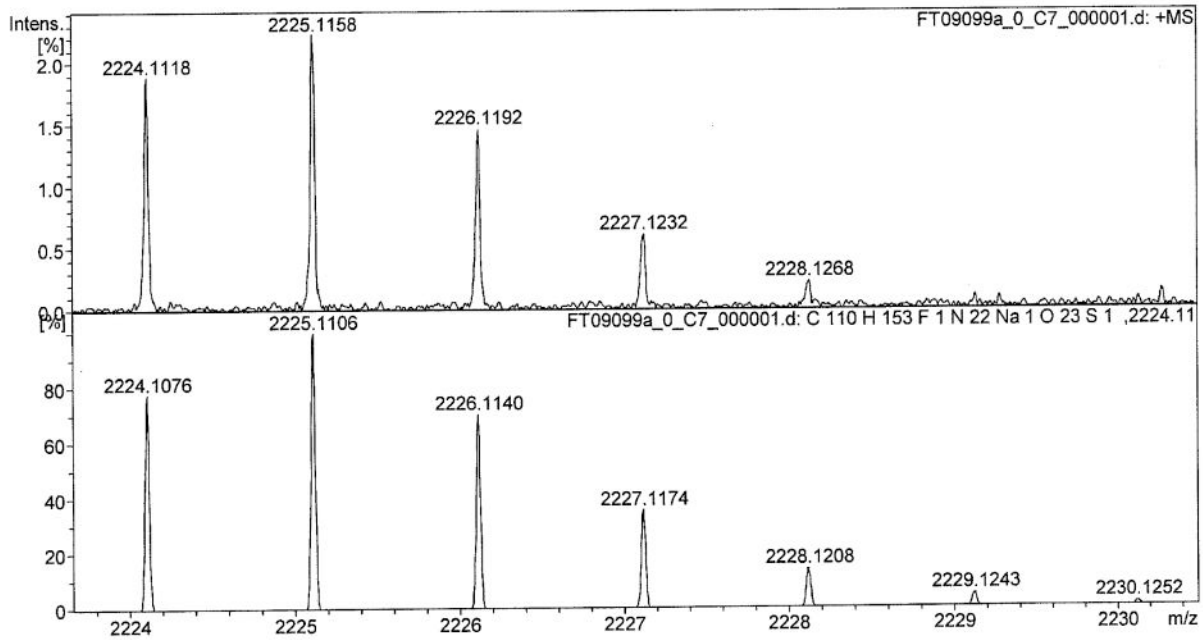


Lasso peptide S37

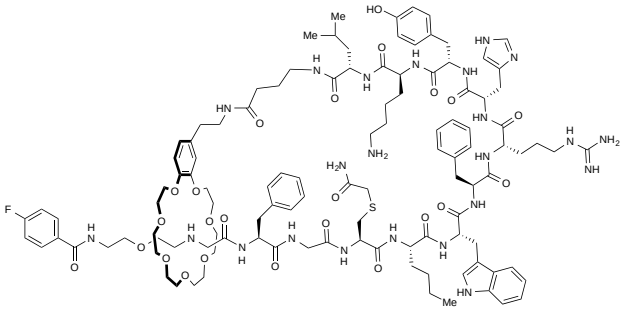


Box 1: Full scale spectra with internal reference peaks (ESI: Na-PFHA 1952.7834, 2724.7011, 3110.6599, 4268.5365, 4654.4953); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.

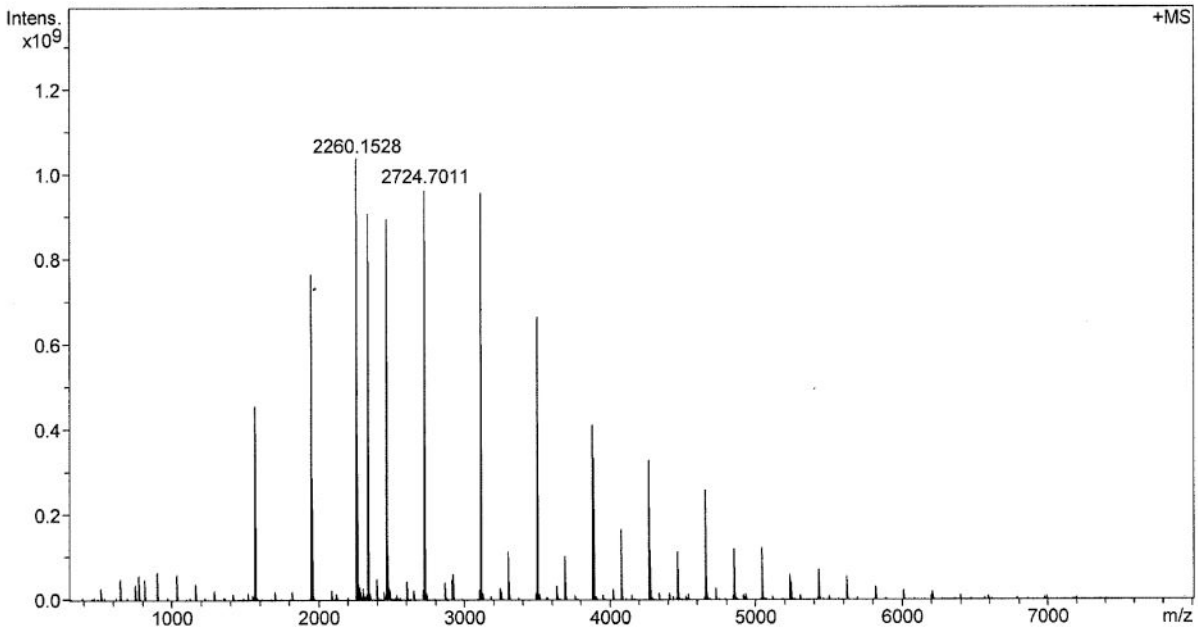


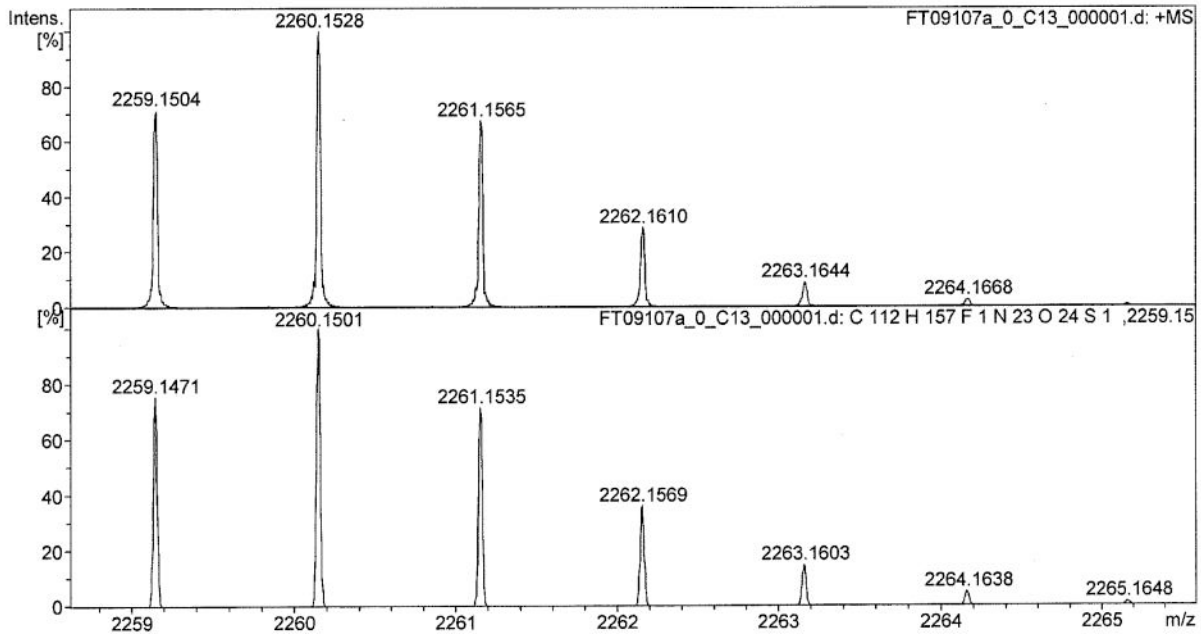


Cys-alkylated lasso peptide 10

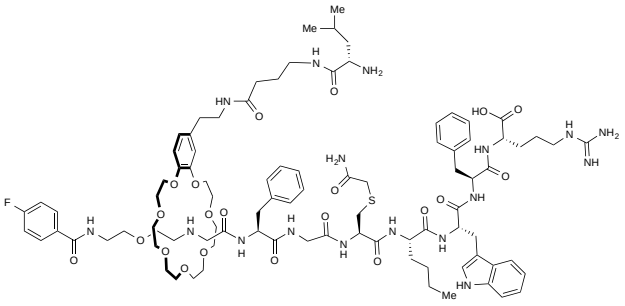


Box 1: Full scale spectra with internal reference peaks (ESI: Na-PFHA 1952.7834, 2724.7011, 3110.6599, 4268.5365, 5426.4130); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.

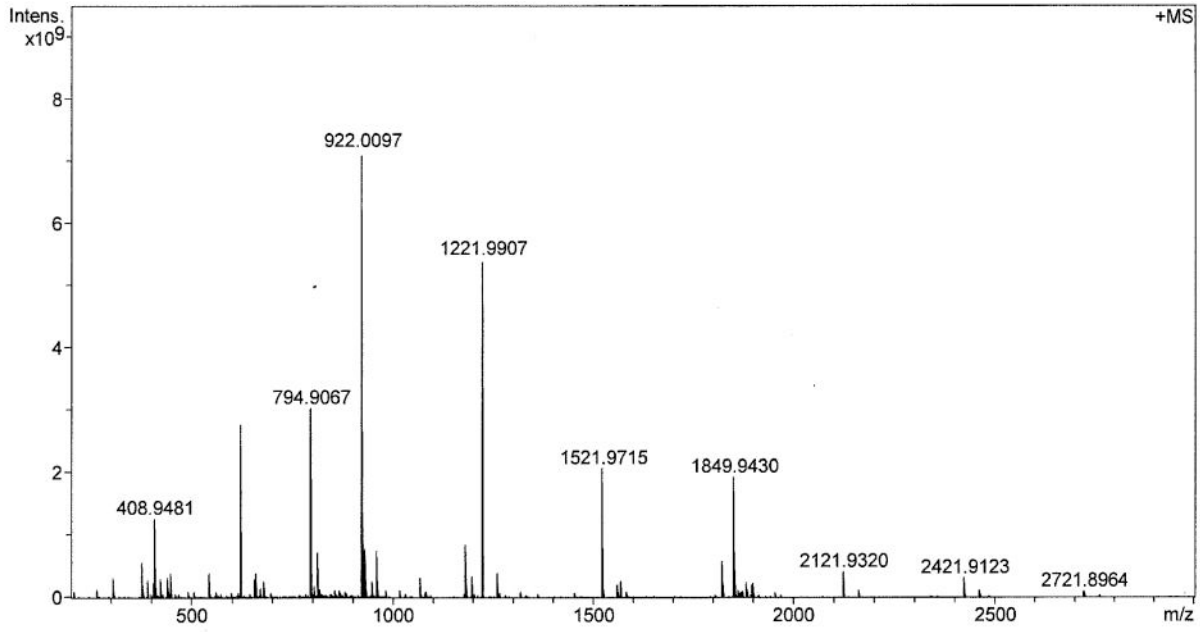


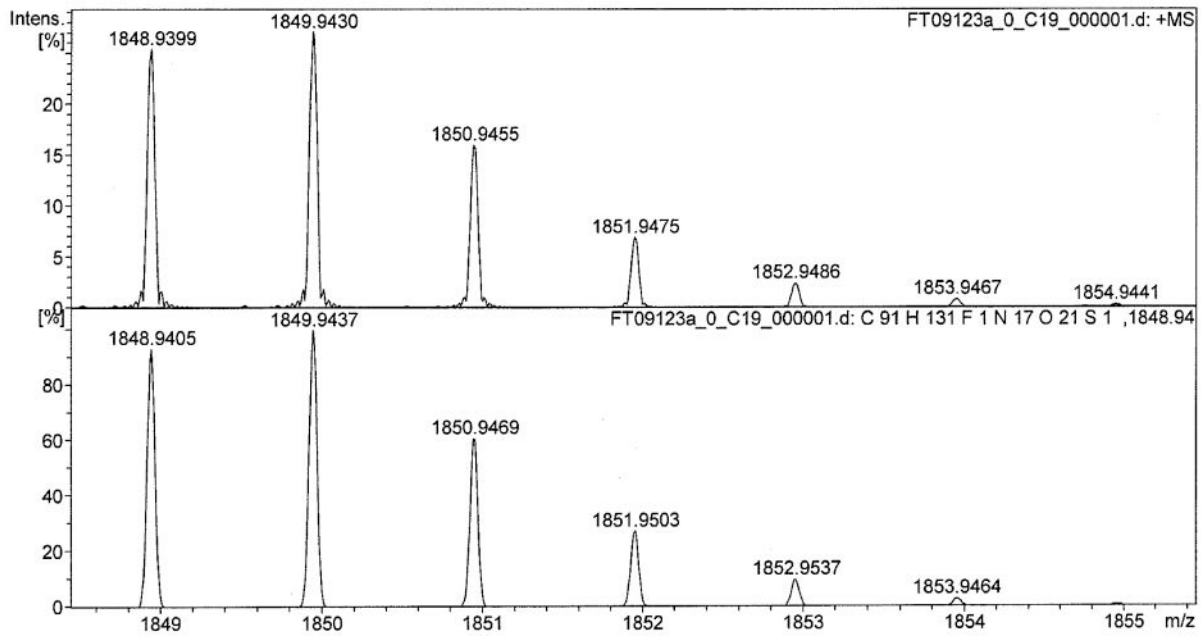


Trypsin digestion product S38

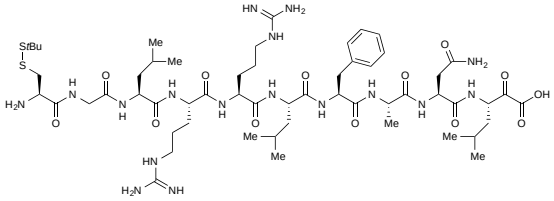


Box 1: Full scale spectra with internal reference peaks (ESI: Na-PFHA DCTBTmix 322.0481, 408.9481, 622.0290, 794.9069, 922.0098, 1180.8657, 1221.9906, 1521.9715, 1566.8246, 1821.9523, 1952.7834, 2338.7423); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.

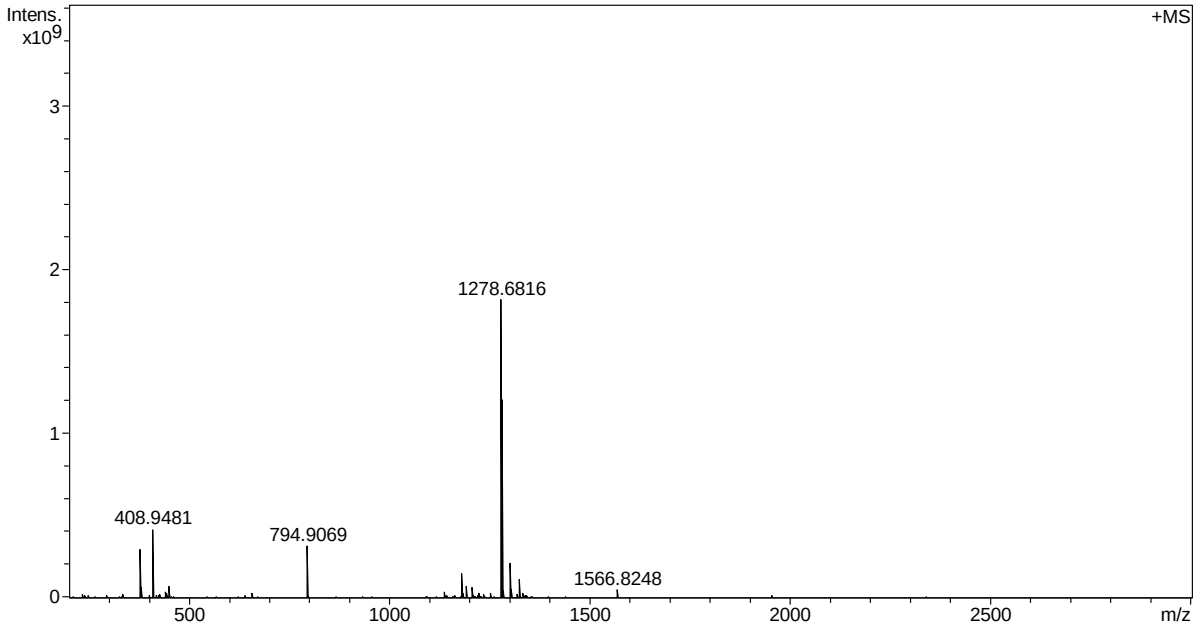


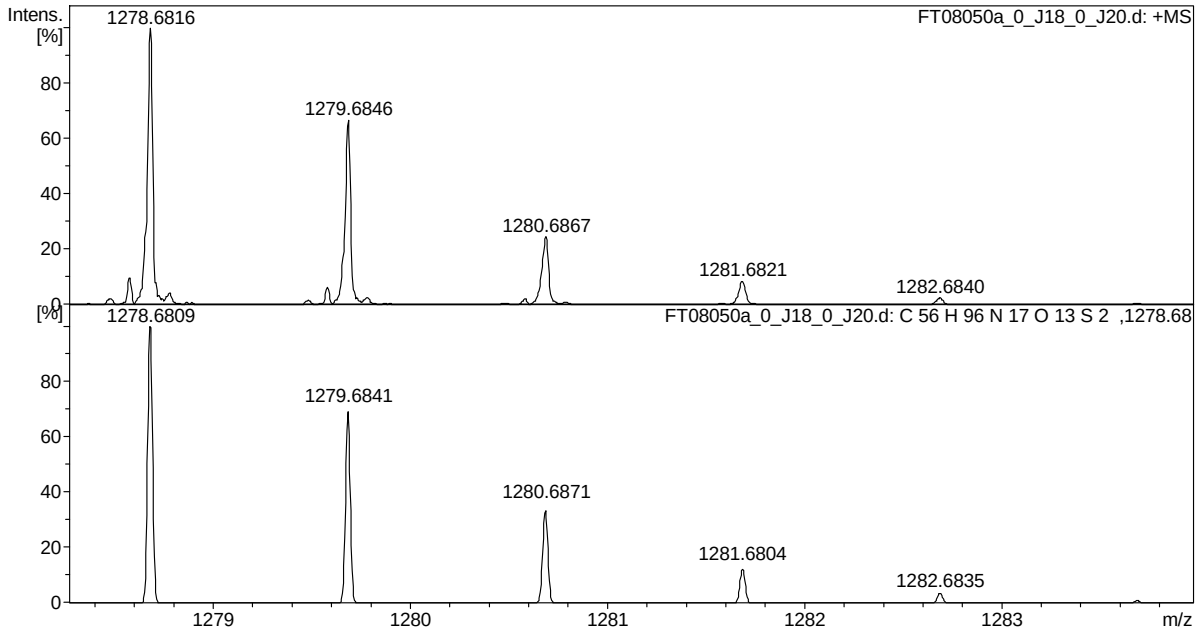


Peptide α-ketoacid lassomycin S39

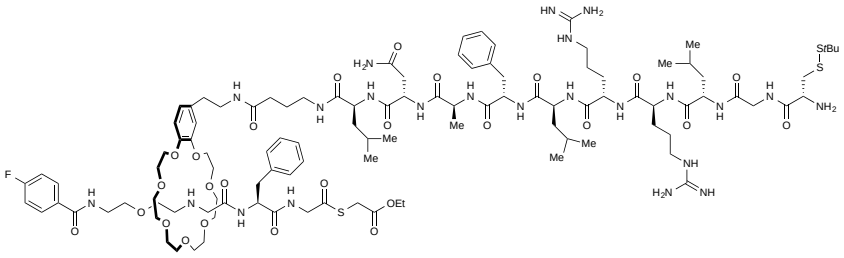


Box 1: Full scale spectra with internal reference peaks (ESI: Na-PFHA 408.9481, 794.9069, 1180.8657, 1566.8246, 1952.7834); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.

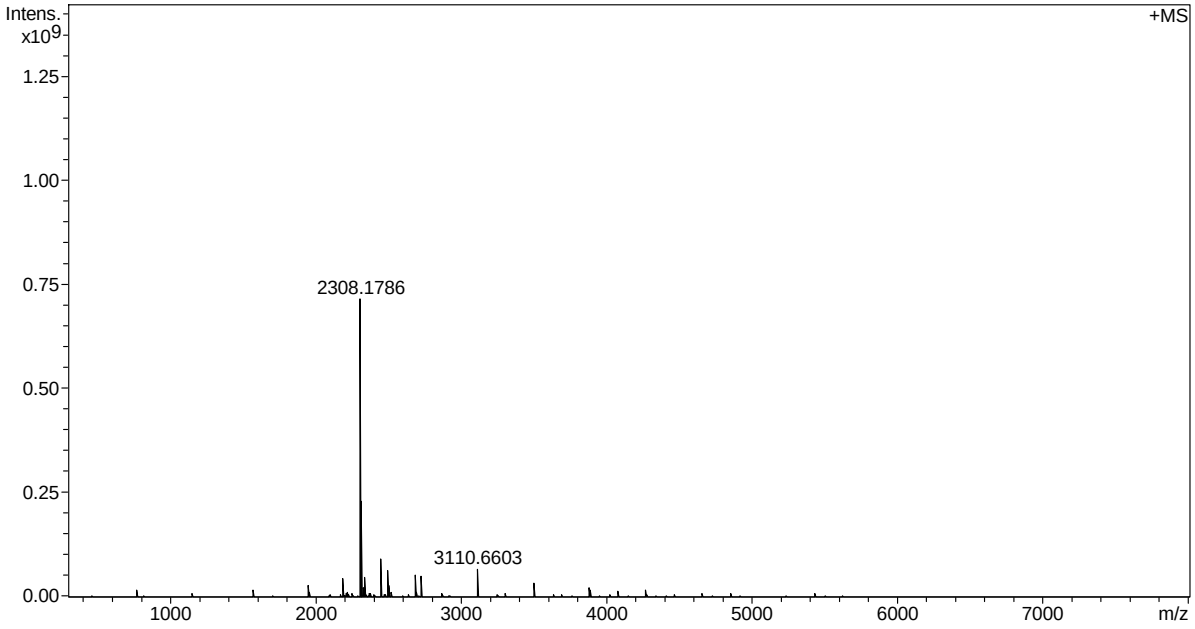


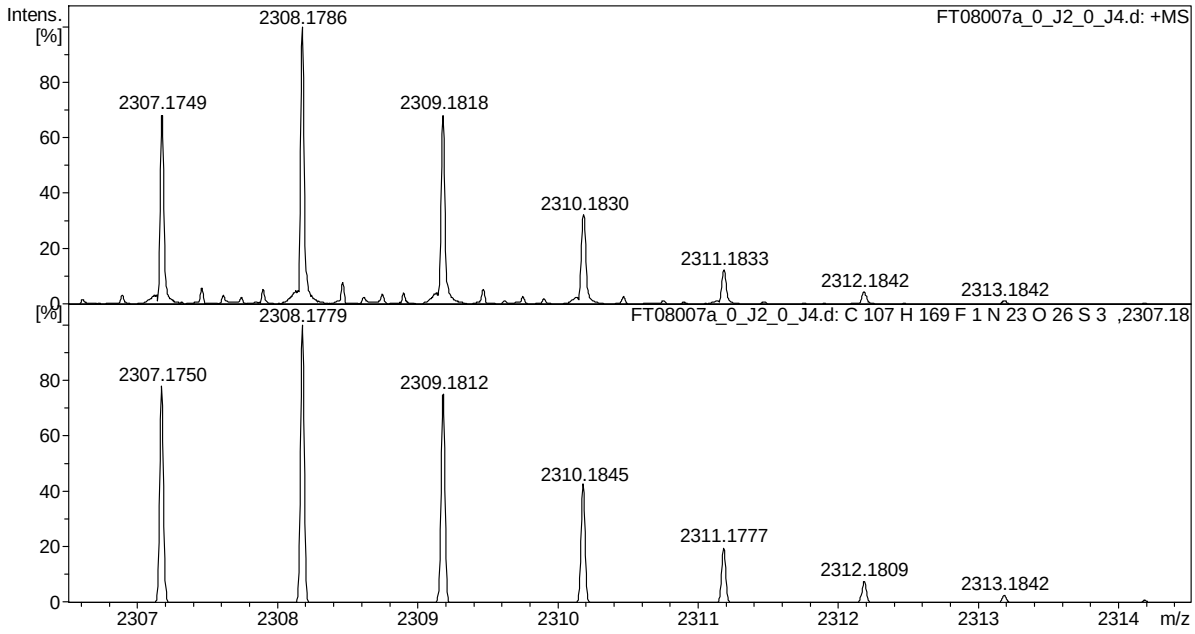


Peptido[2]rotaxane S40

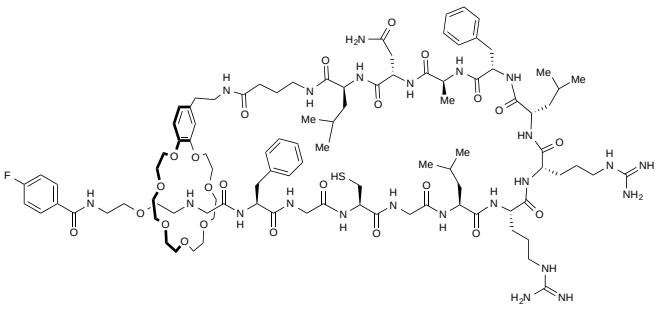


Box 1: Full scale spectra with internal reference peaks (ESI: Na-PFHA 1952.7834, 2724.7011, 3110.6599, 3496.6188); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.

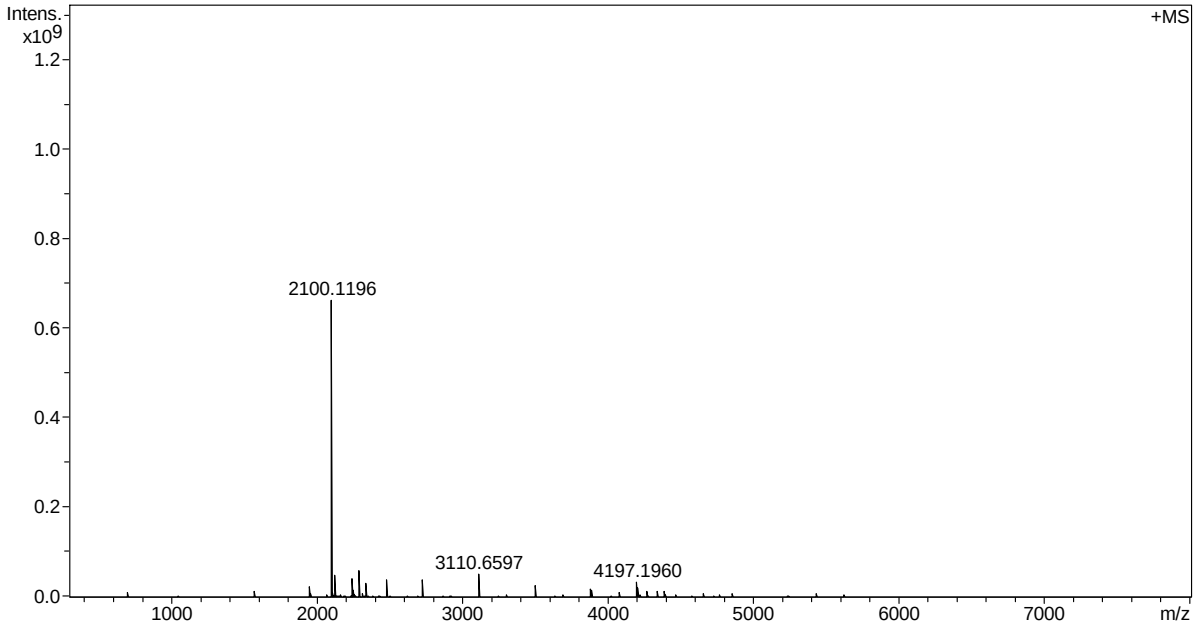


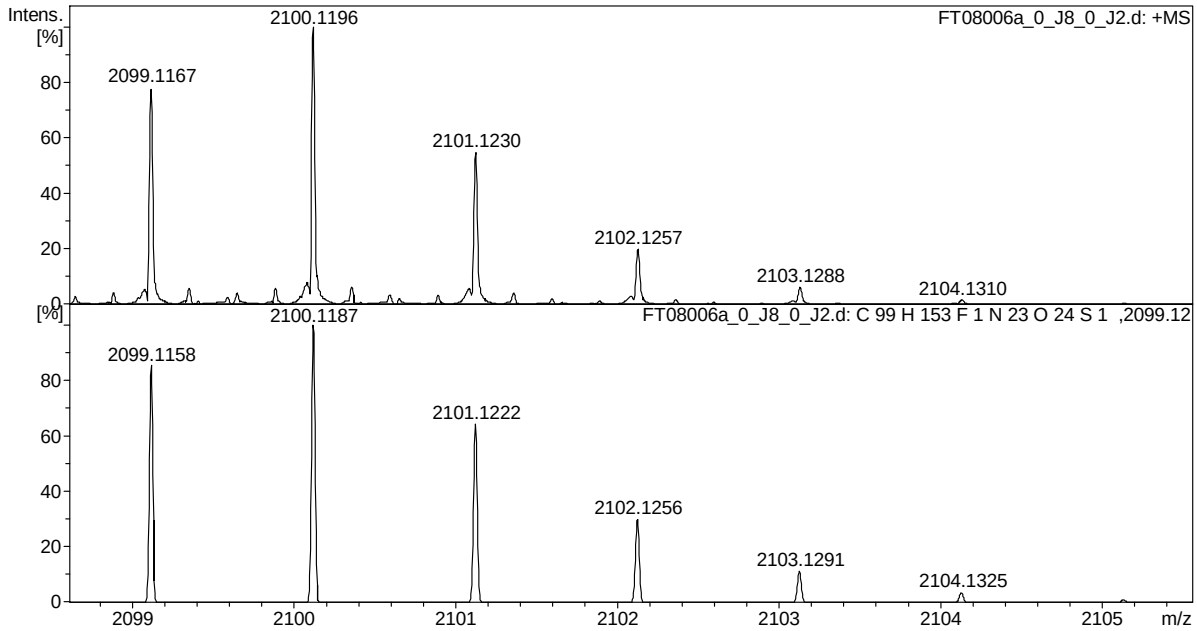


Lasso peptide S41

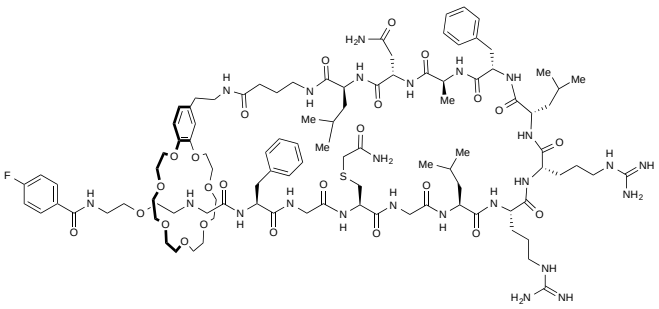


Box 1: Full scale spectra with internal reference peaks (ESI: Na-PFHA 1952.7834, 2338.7423, 2724.7011, 3110.6599); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.

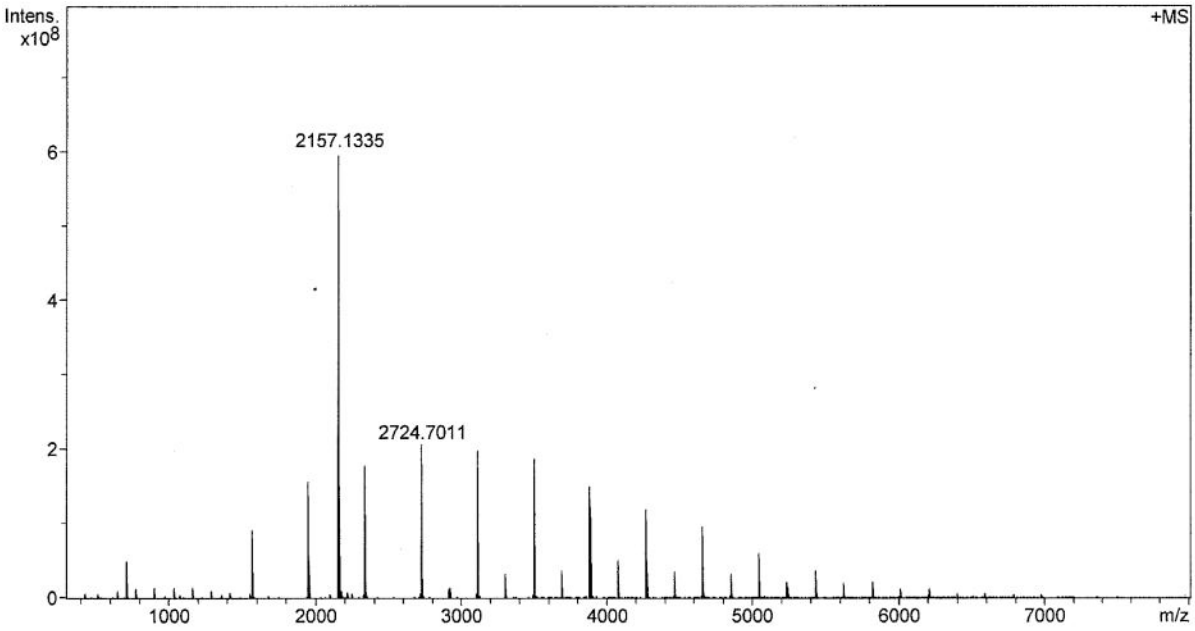


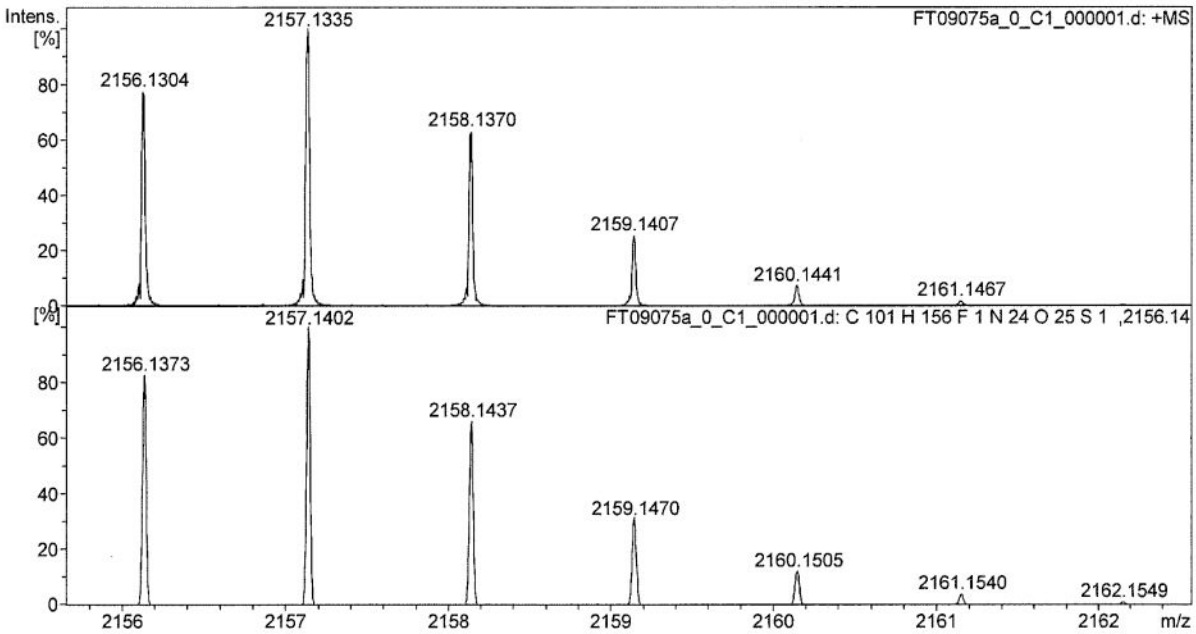


Cys-alkylated lasso peptide 11

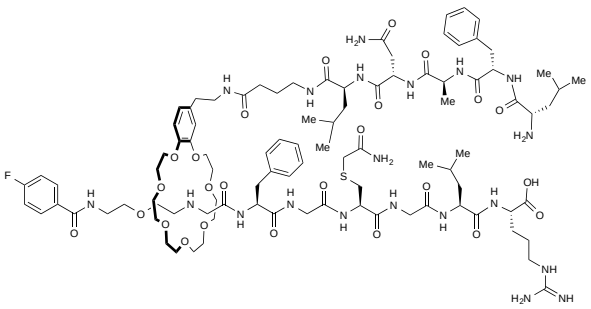


Box 1: Full scale spectra with internal reference peaks (ESI: Na-PFHA 1566.8246, 1952.7834, 2724.7011, 3110.6599); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.

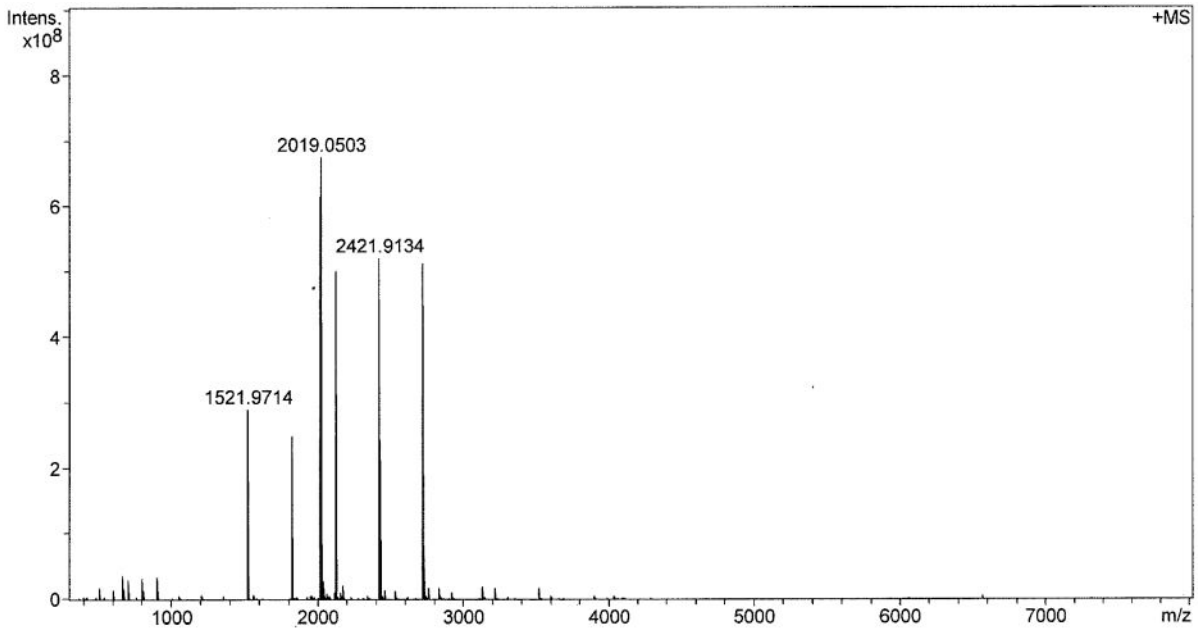


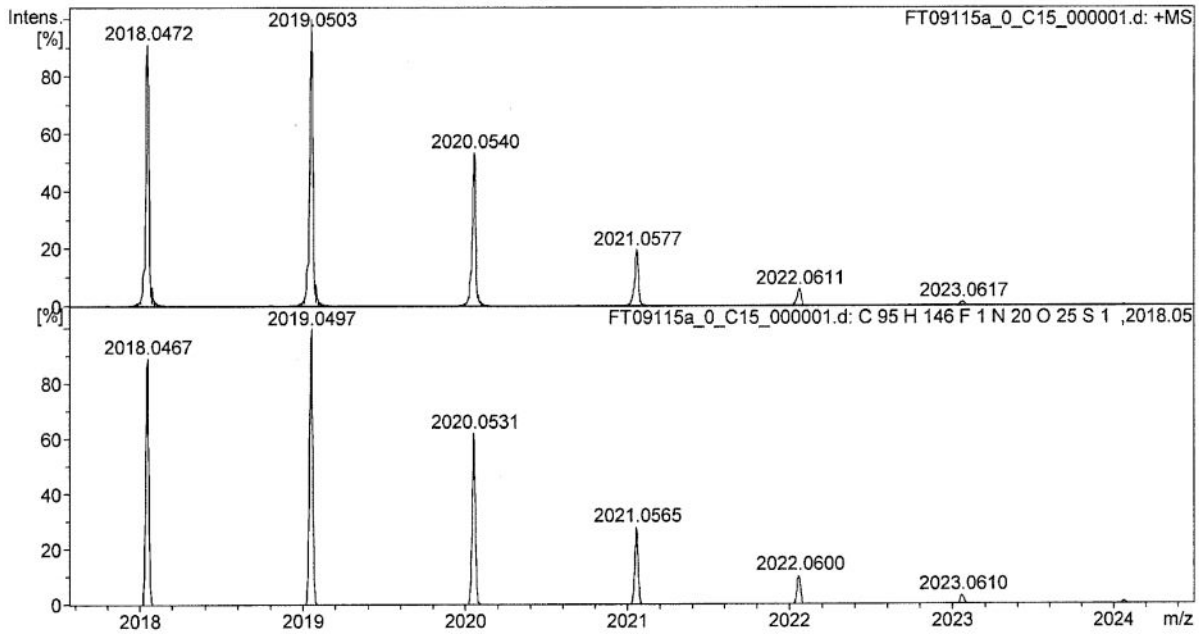


Trypsin digestion product S42

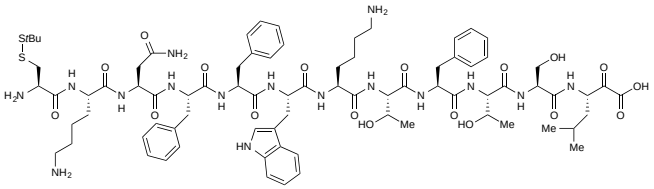


Box 1: Full scale spectra with internal reference peaks (ESI: Tuning Mix ES-TOF (ESI) (pos)DCTB 1521.9715, 1821.9523, 2121.9332, 2421.9140, 2721.8948); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.

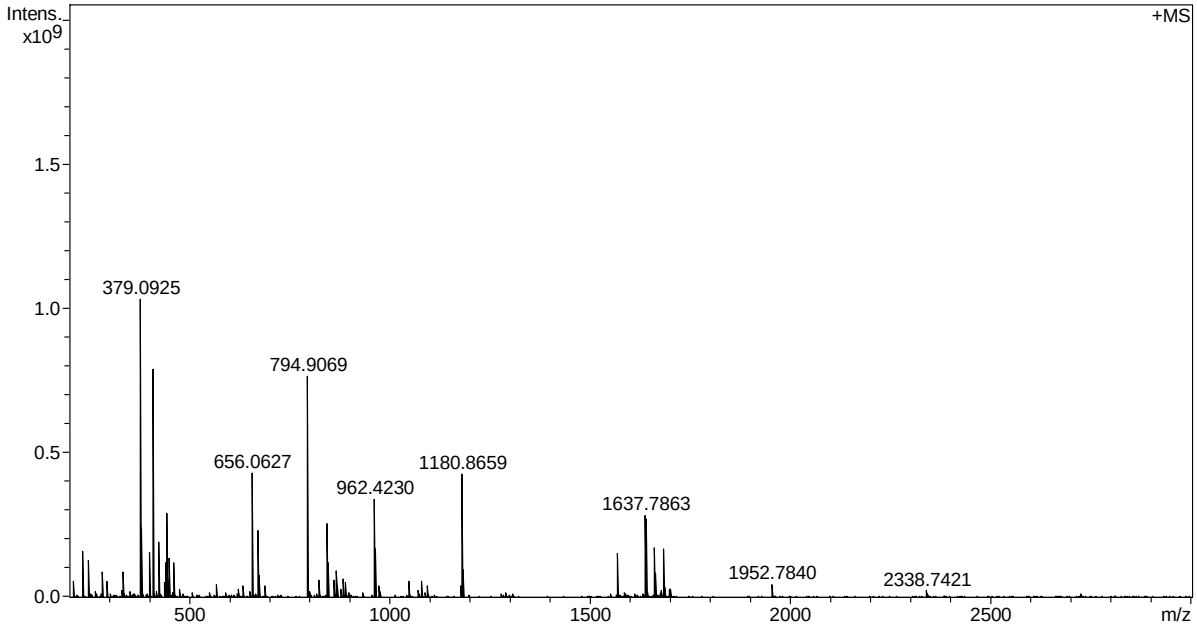


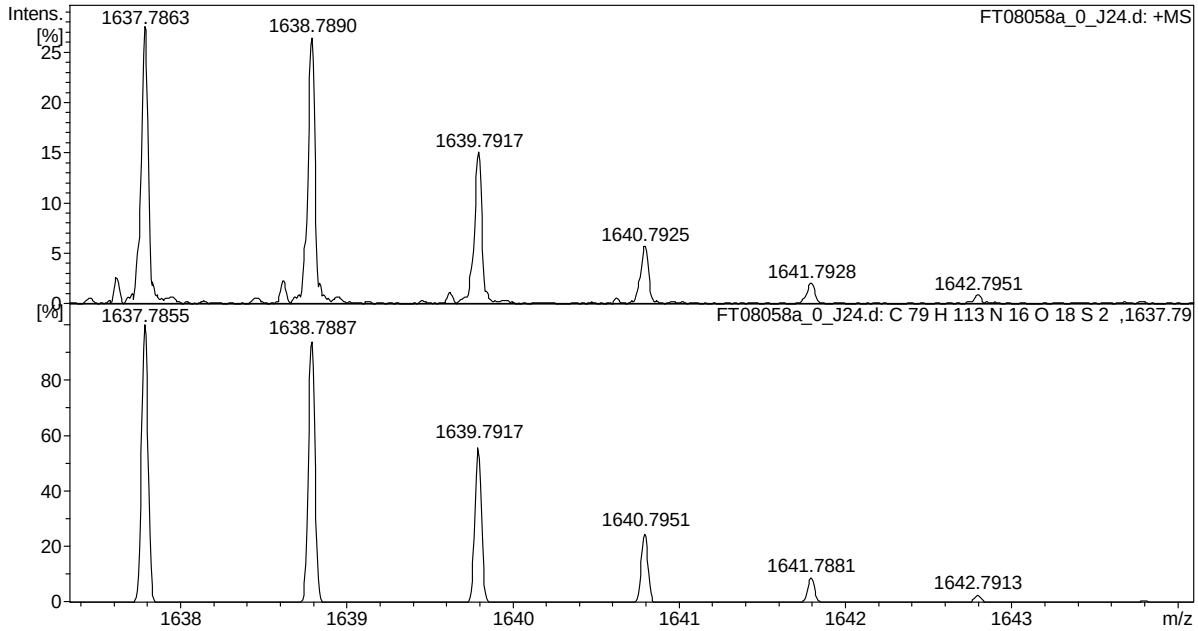


Peptide α-ketoacid somatostatin S43

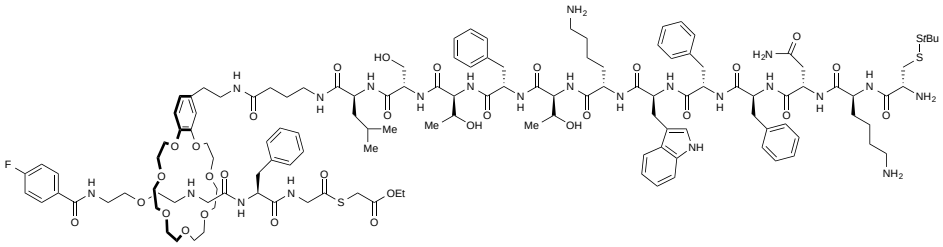


Box 1: Full scale spectra with internal reference peaks (ESI: Na-PFHA 408.9481, 794.9069, 1180.8657, 1566.8246, 1952.7834); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.

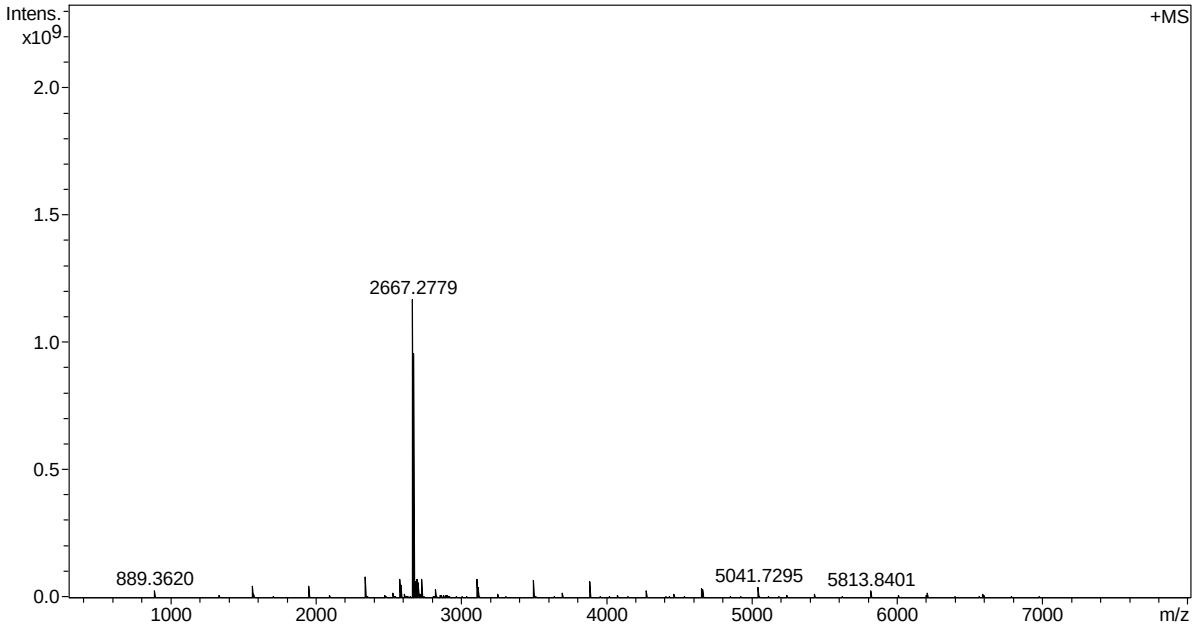


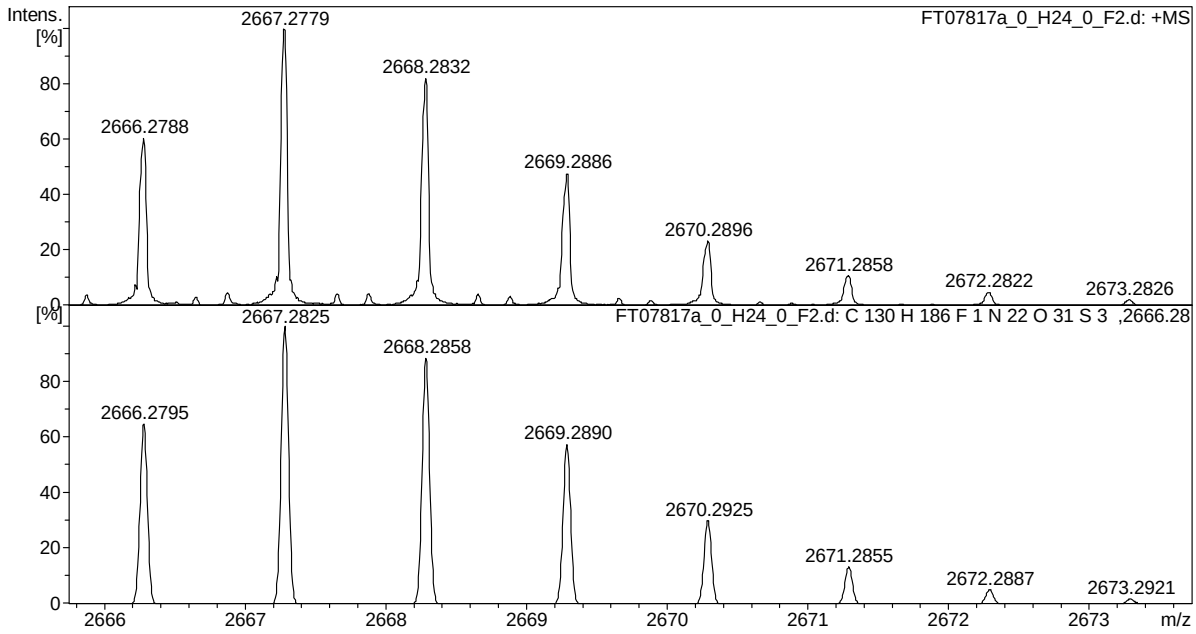


Peptido[2]rotaxane S44

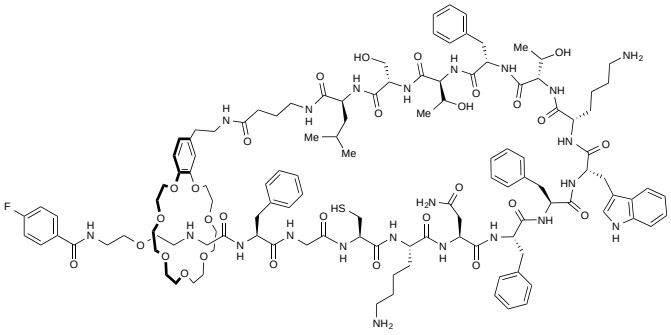


Box 1: Full scale spectra with internal reference peaks (ESI: Na-PFHA 2338.7423, 2724.7011, 3110.6599, 3882.5776); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.

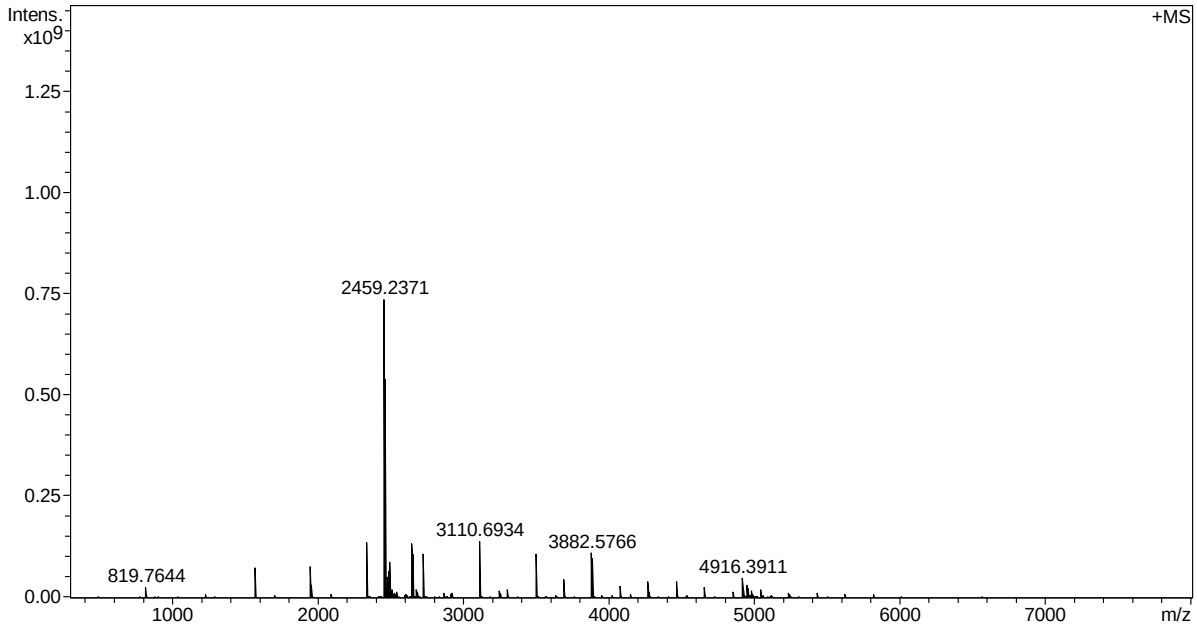


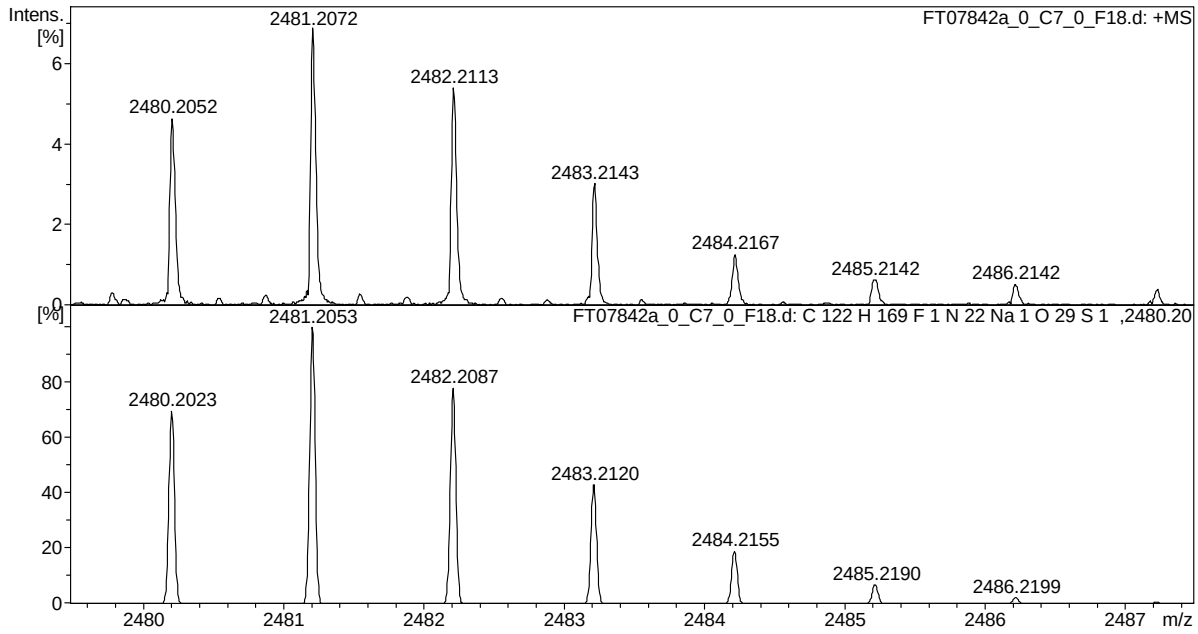


Lasso peptide S45

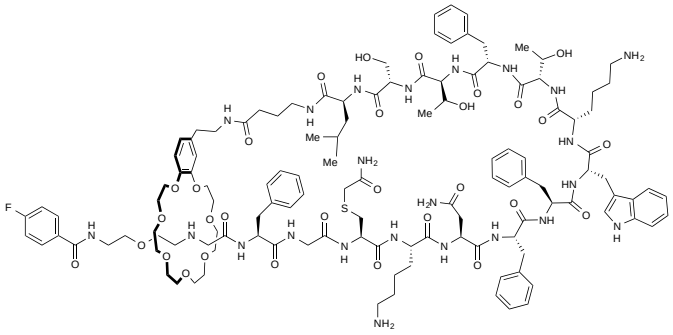


Box 1: Full scale spectra with internal reference peaks (ESI: Na-PFHA 2338.7423, 2724.7011, 3496.6188, 3882.5776); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.

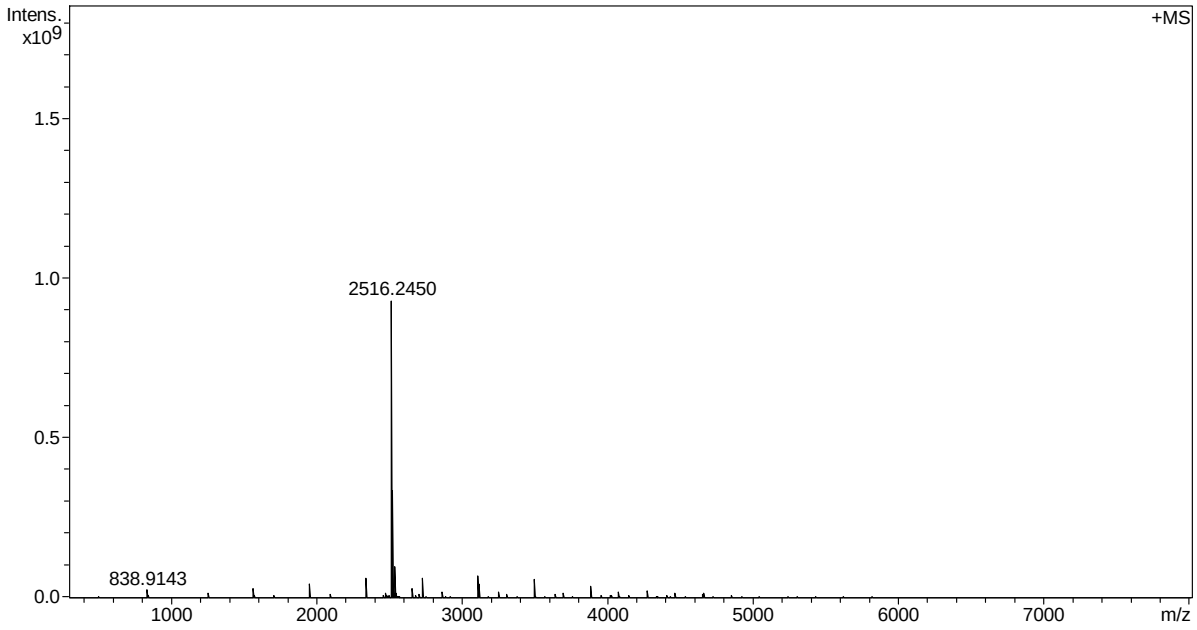


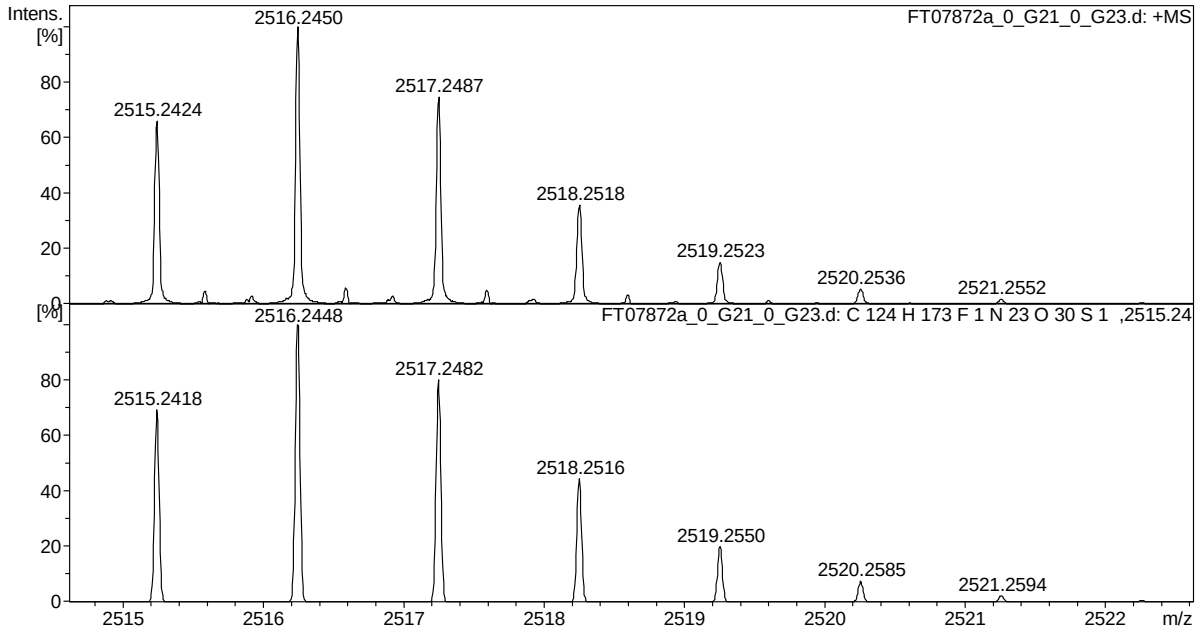


Cys-alkylated lasso peptide 12

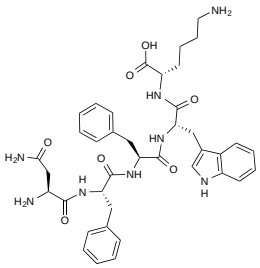


Box 1: Full scale spectra with internal reference peaks (ESI: Na-PFHA 2338.7423, 2724.7011, 3110.6599, 3496.6188); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.

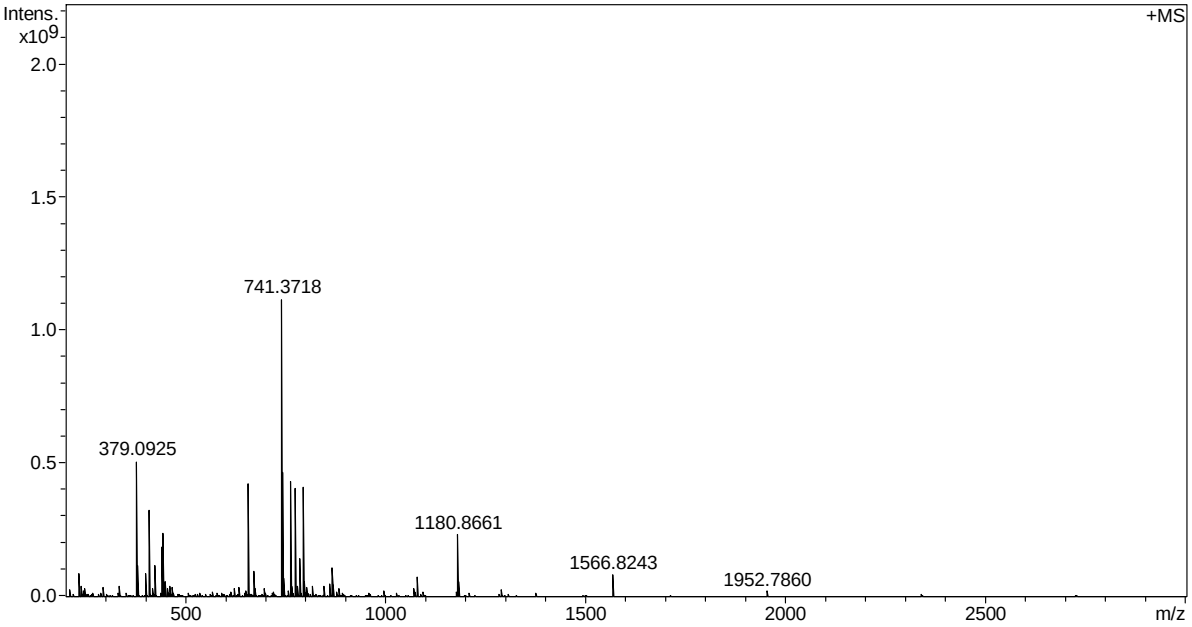


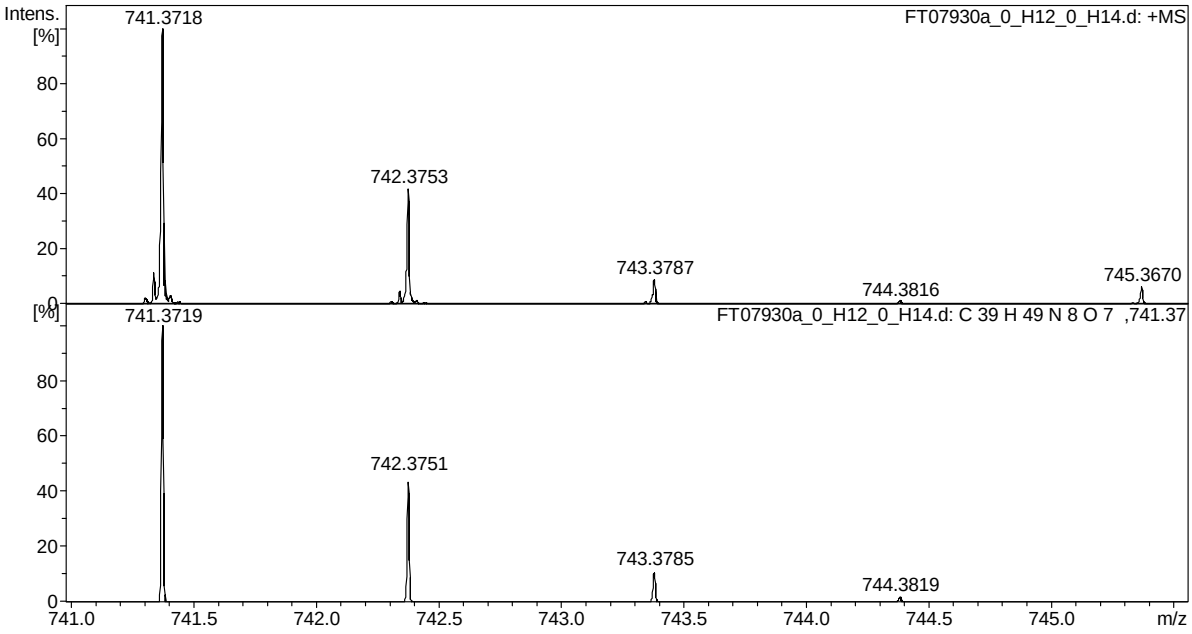


Degradation product S46

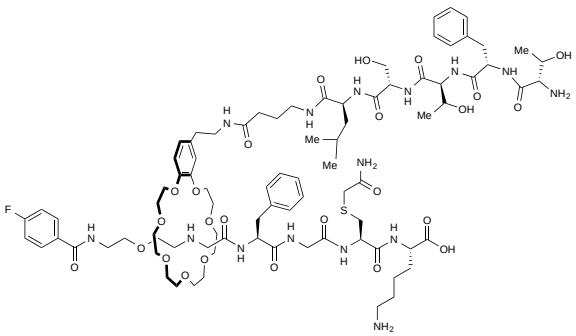


Box 1: Full scale spectra with internal reference peaks (ESI: Na-PFHA 408.9481, 794.9069, 1180.8657, 1566.8246); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.

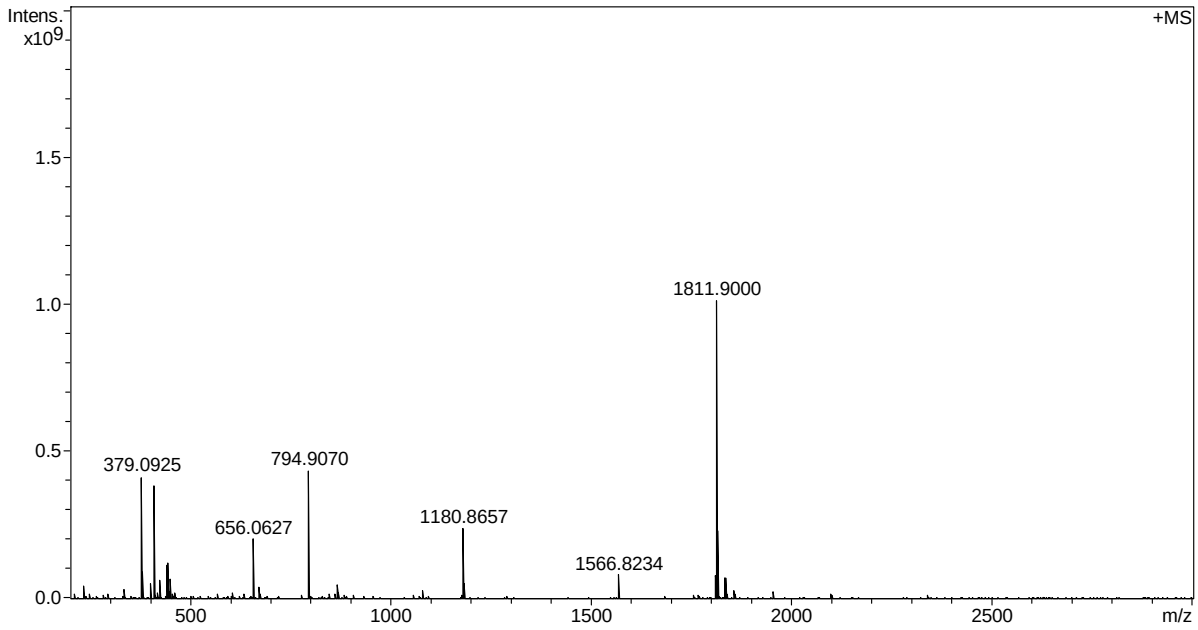


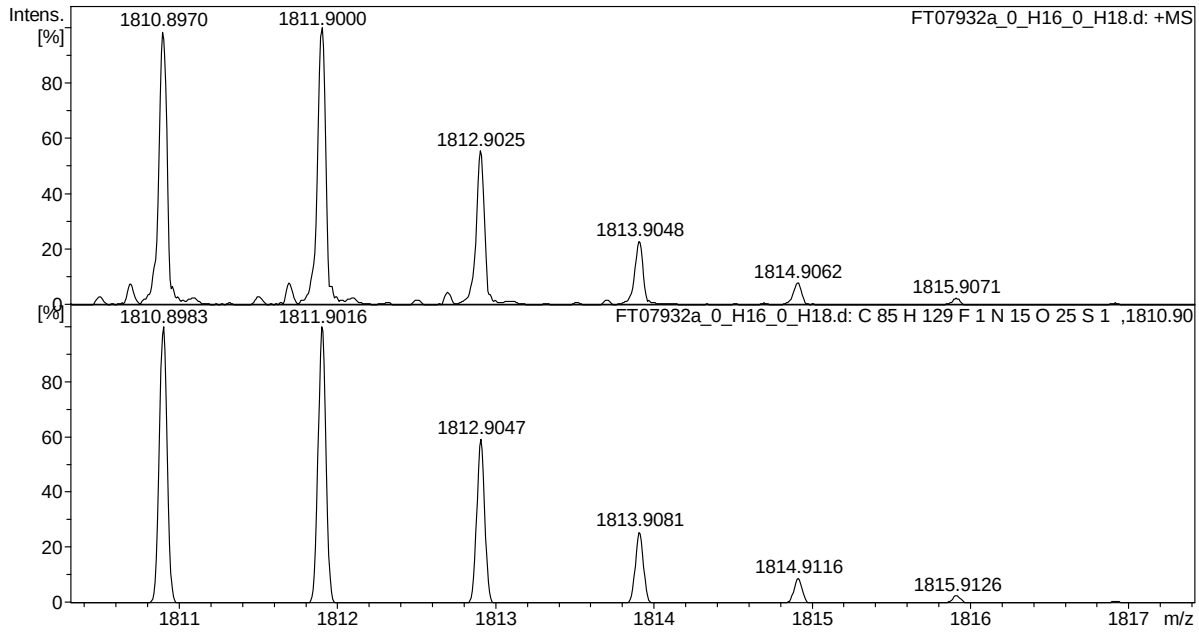


Degradation product S47

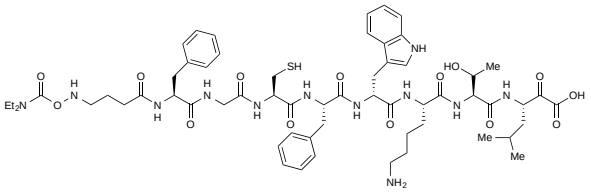


Box 1: Full scale spectra with internal reference peaks (ESI: Na-PFHA 408.9481, 794.9069, 1180.8657, 1566.8246, 1952.7834); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.

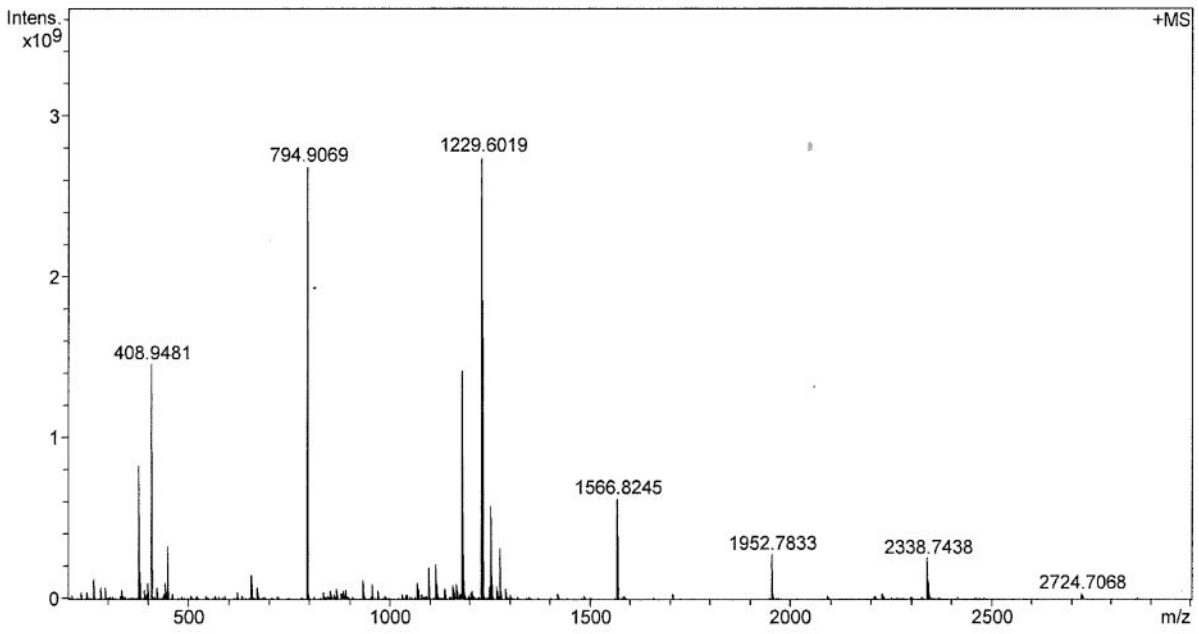


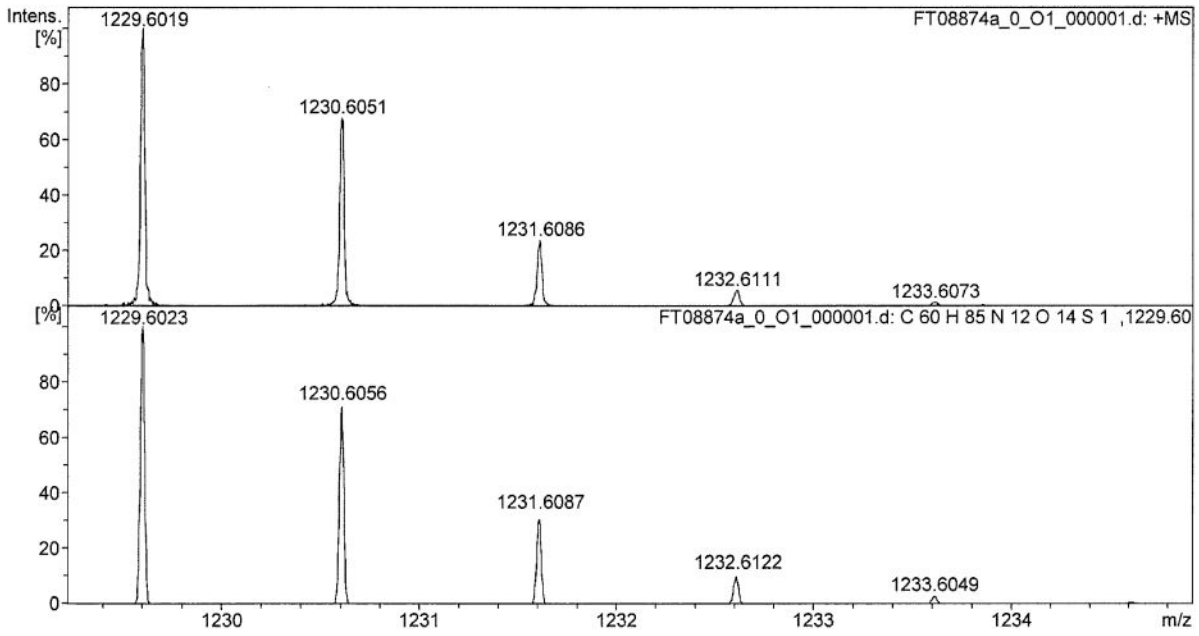


Bifunctional linear peptide S48

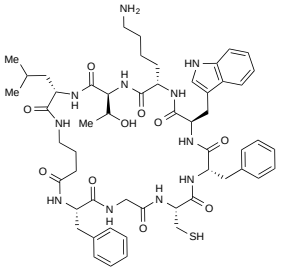


Box 1: Full scale spectra with internal reference peaks (ESI: Na-PFHA 408.9481, 794.9069, 1180.8657, 1566.8246); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.

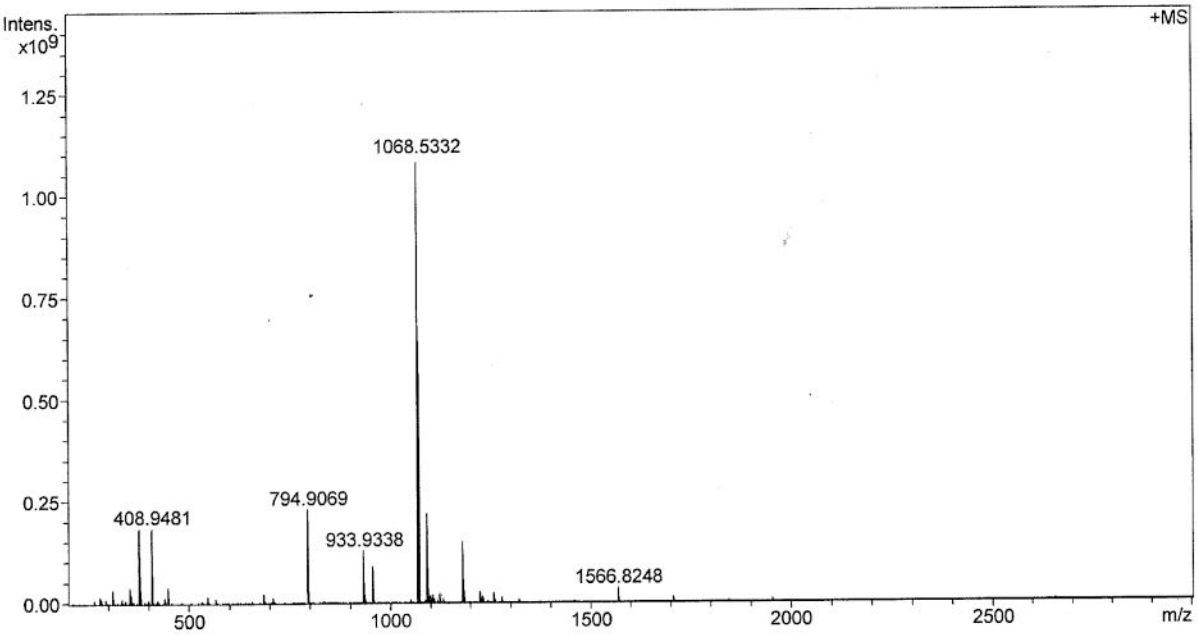


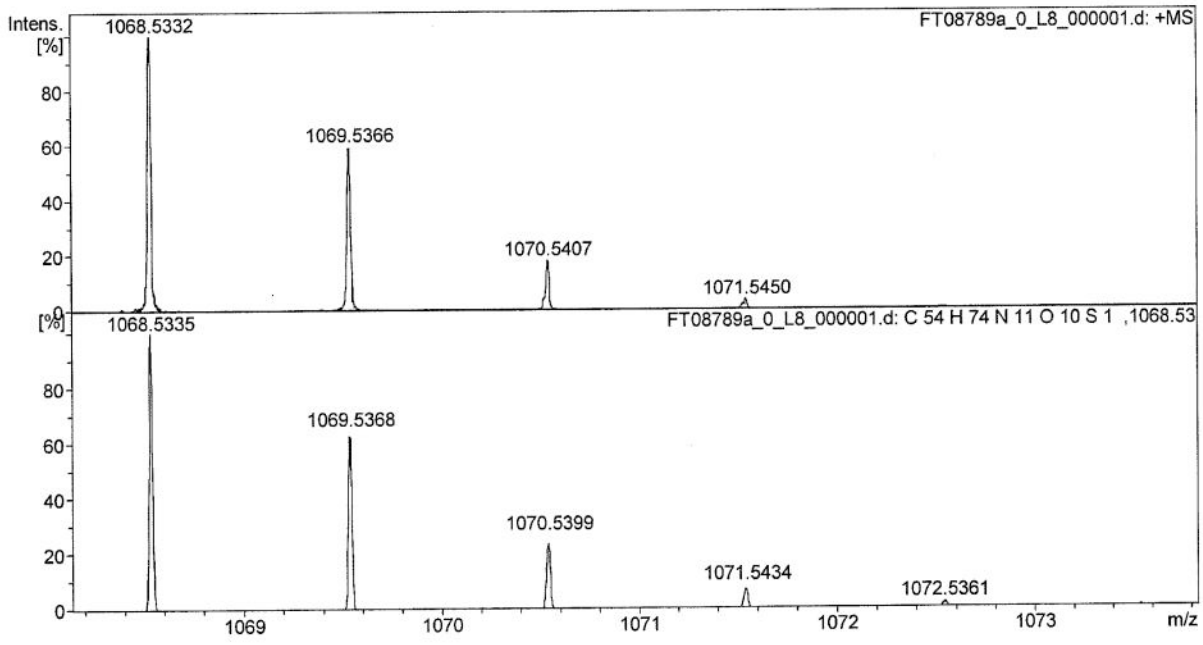


Cyclic peptide S49

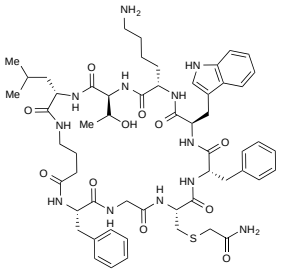


Box 1: Full scale spectra with internal reference peaks (ESI: Na-PFHA 408.9481, 794.9069, 1180.8657, 1566.8246); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.

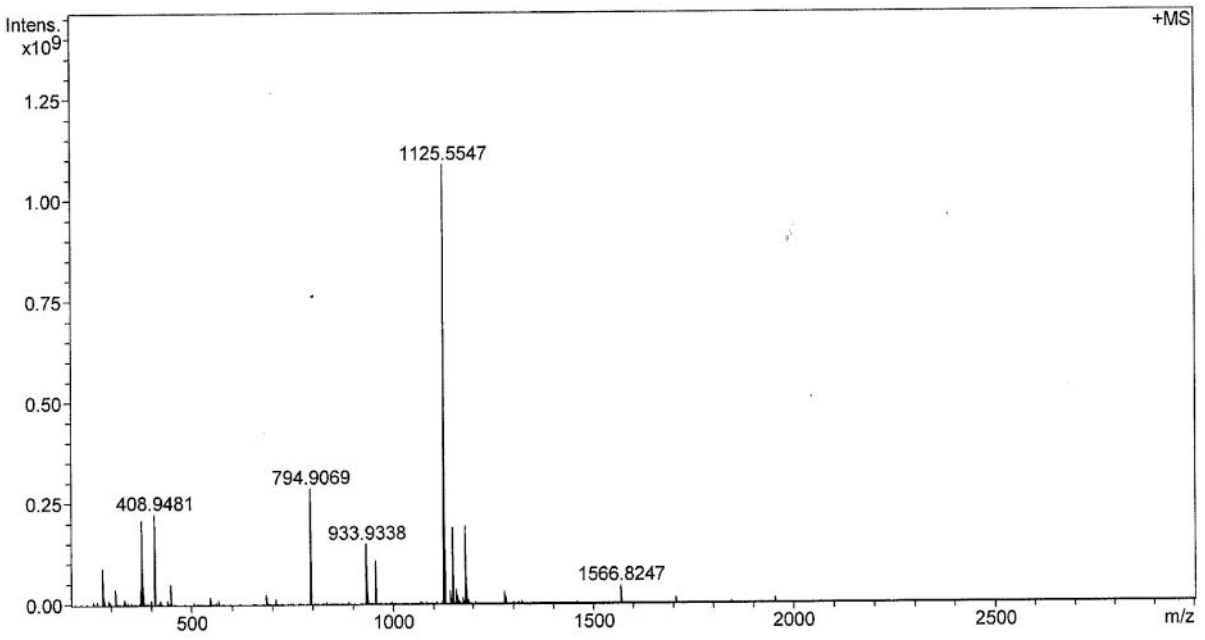


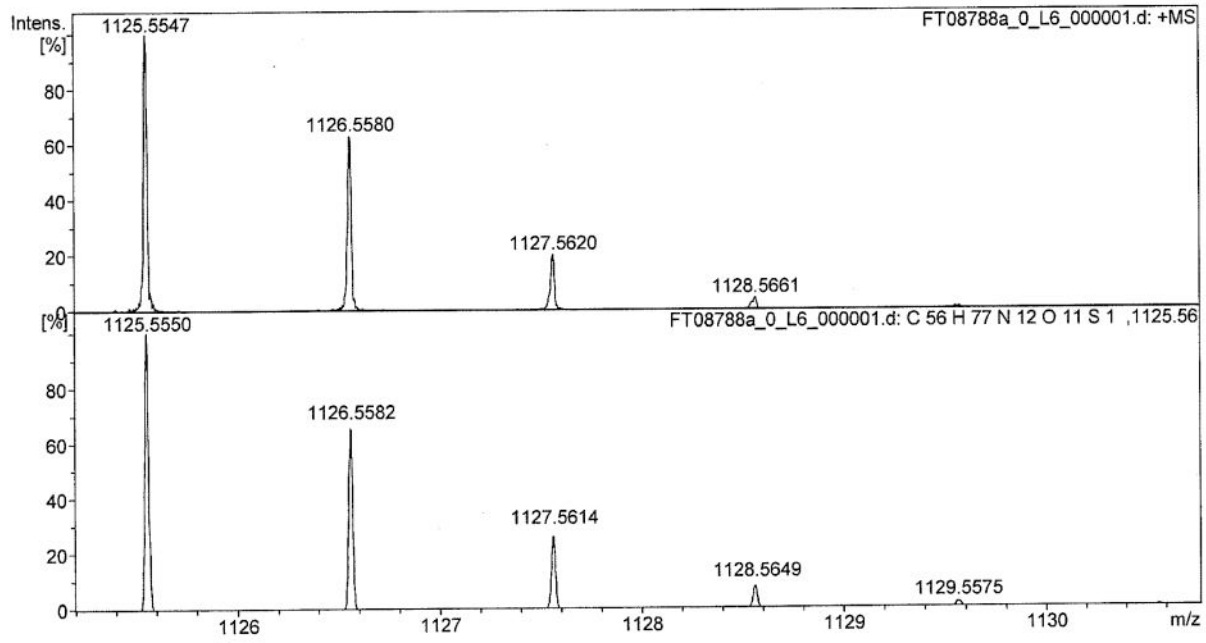


Cyclic peptide C1

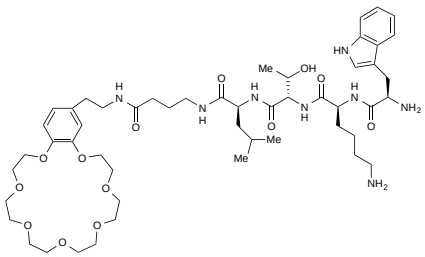


Box 1: Full scale spectra with internal reference peaks (ESI: Na-PFHA 408.9481, 794.9069, 1180.8657, 1566.8246); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.

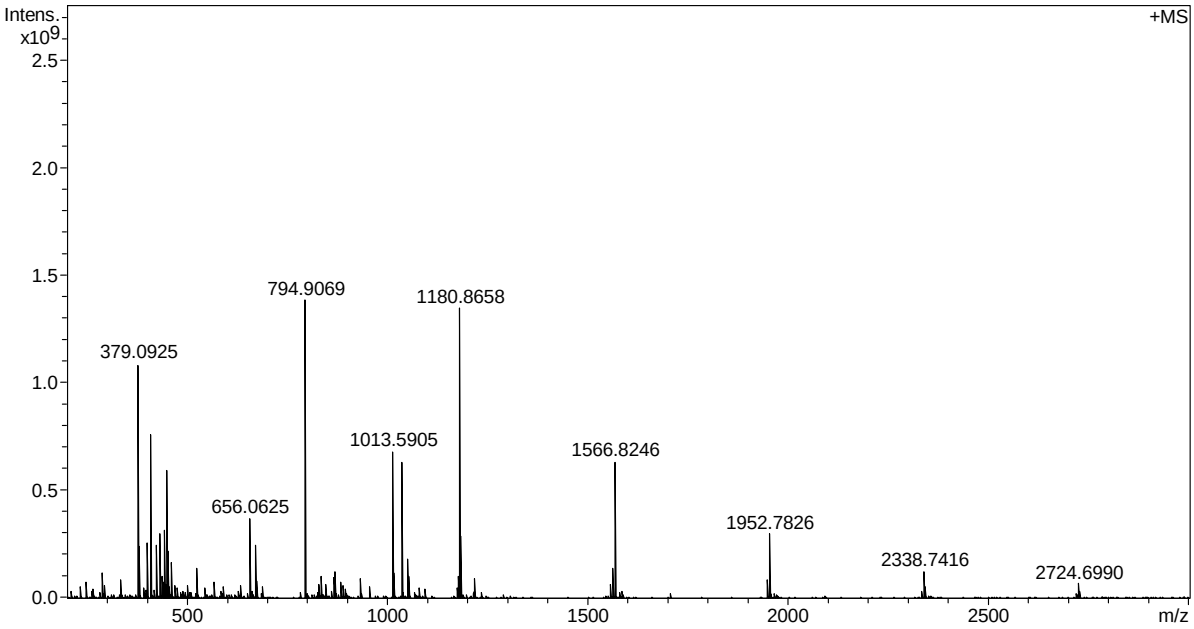


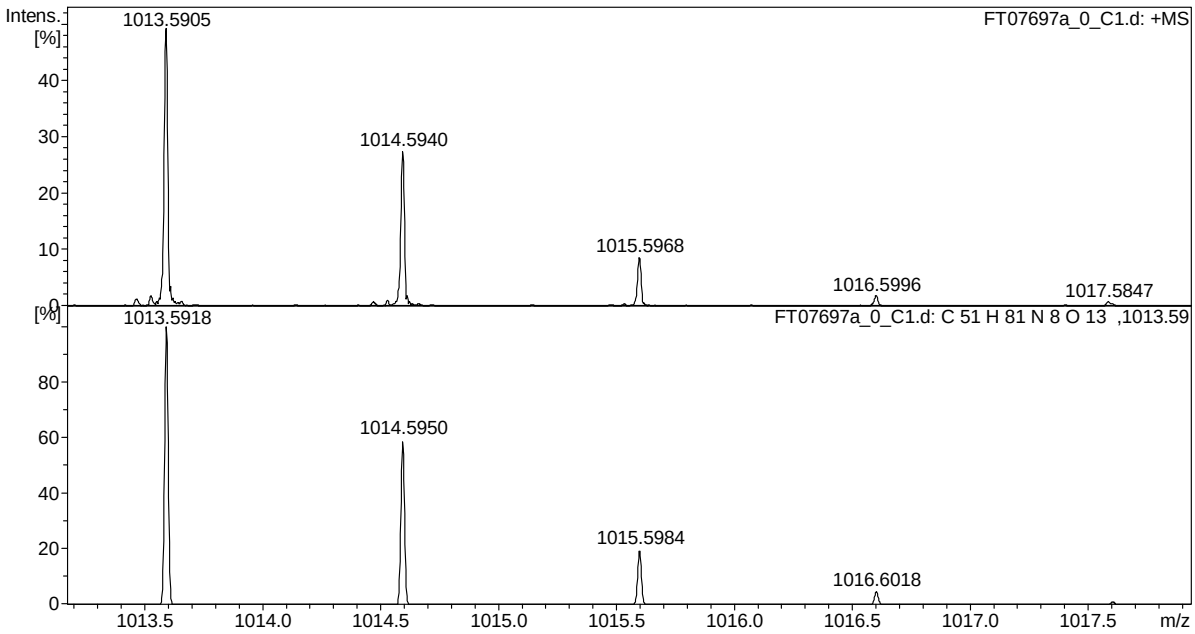


Degradation product S50

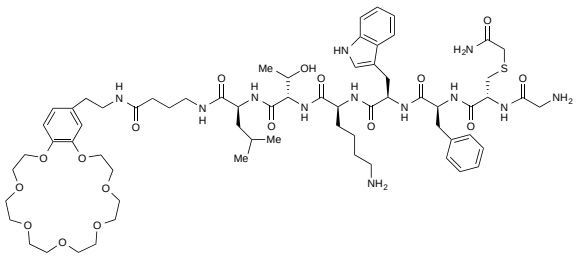


Box 1: Full scale spectra with internal reference peaks (ESI: Na-PFHA 408.9481, 794.9069, 1180.8657, 1566.8246); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.

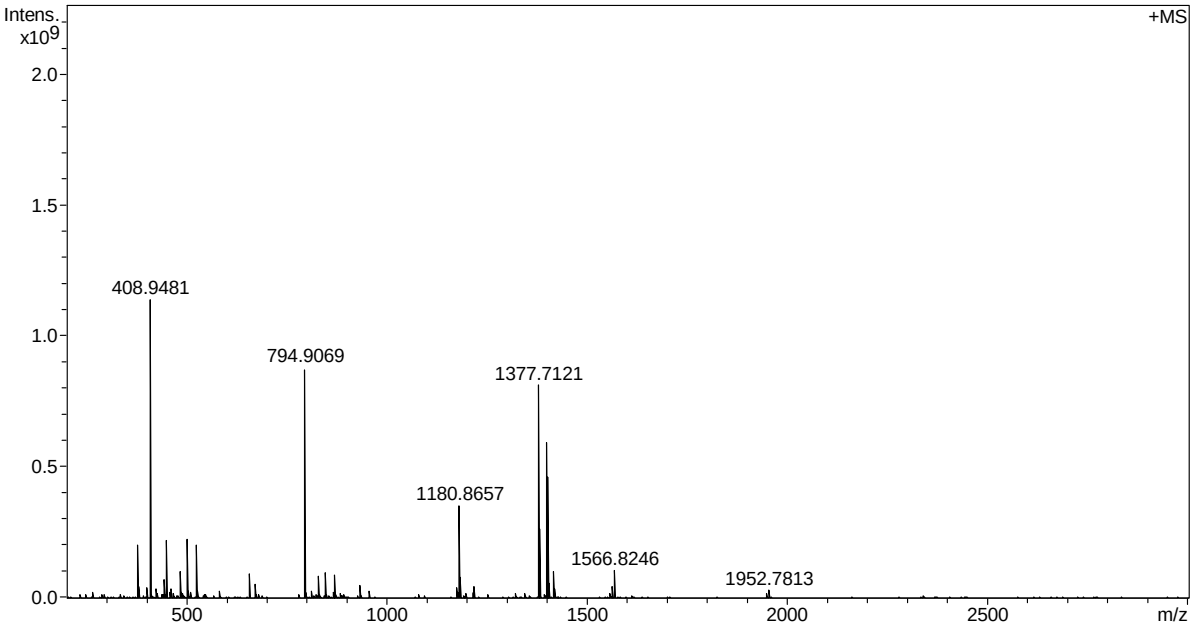


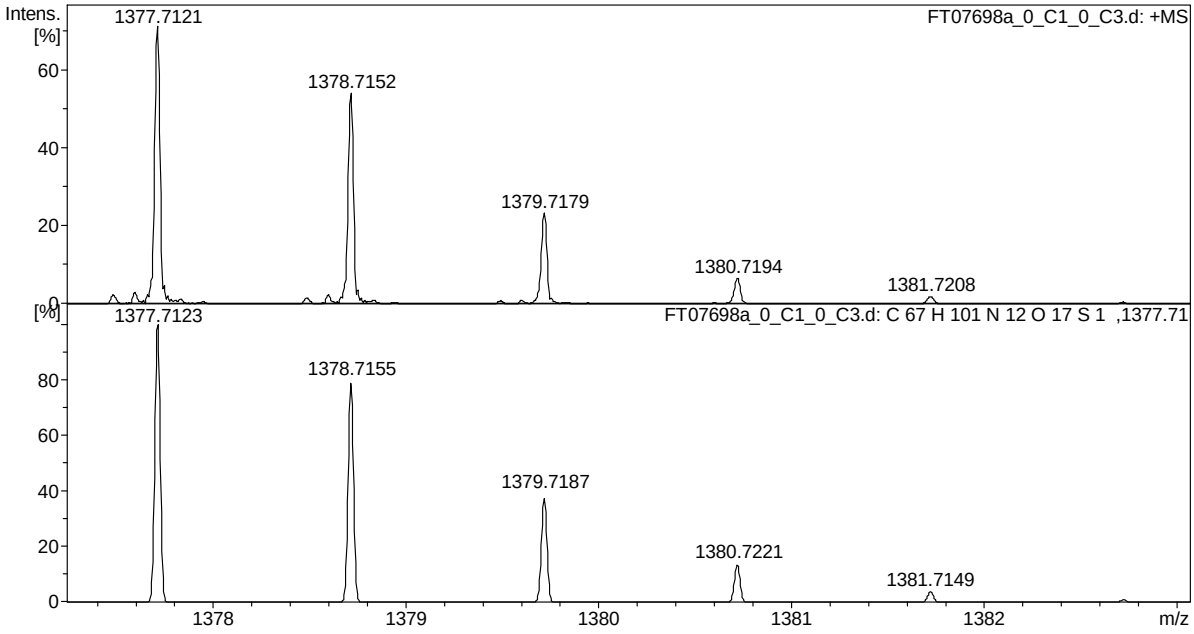


Degradation product S51

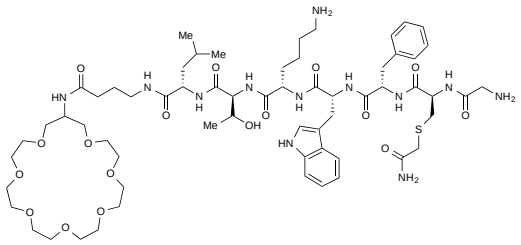


Box 1: Full scale spectra with internal reference peaks (ESI: Na-PFHA 408.9481, 794.9069, 1180.8657, 1566.8246); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.

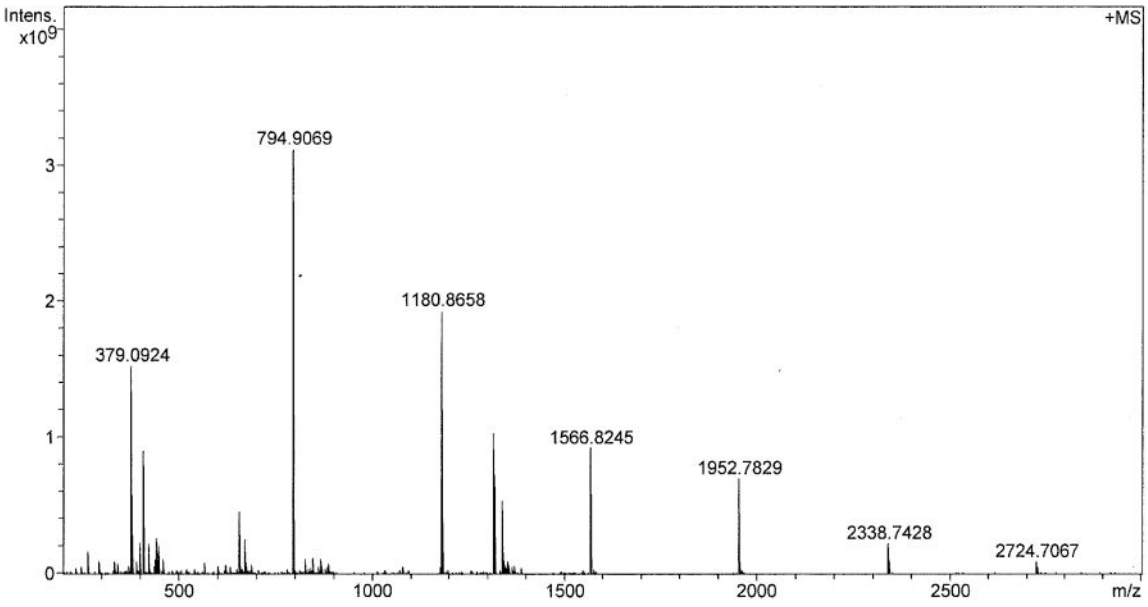


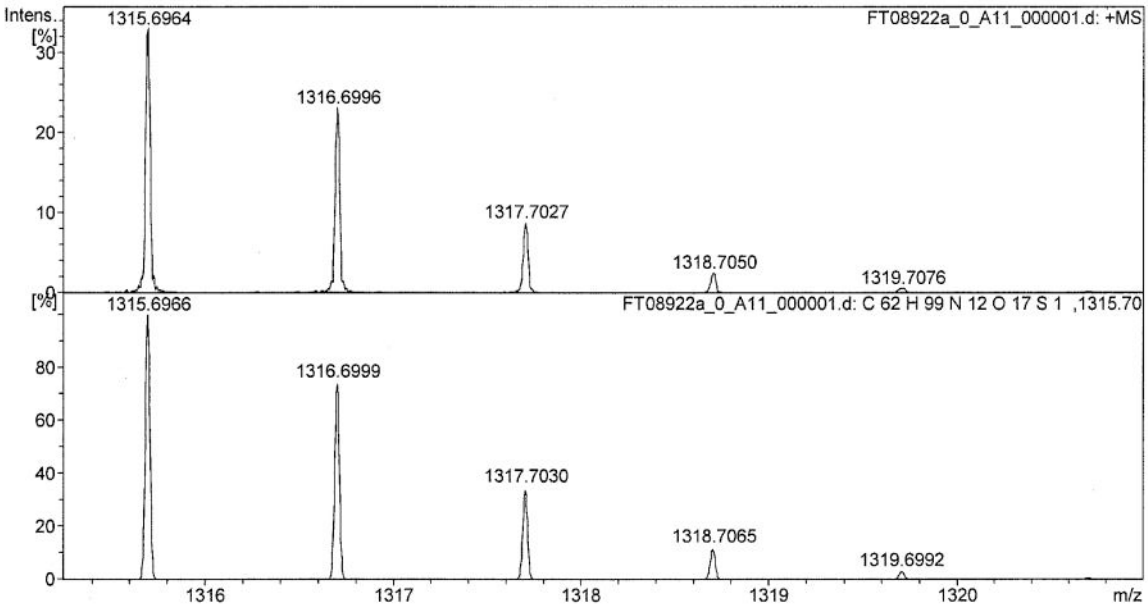


Degradation product S52

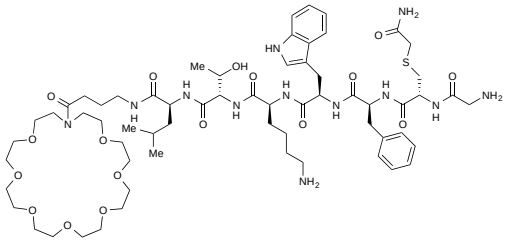


Box 1: Full scale spectra with internal reference peaks (ESI: Na-PFHA 408.9481, 794.9069, 1180.8657, 1566.8246); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.

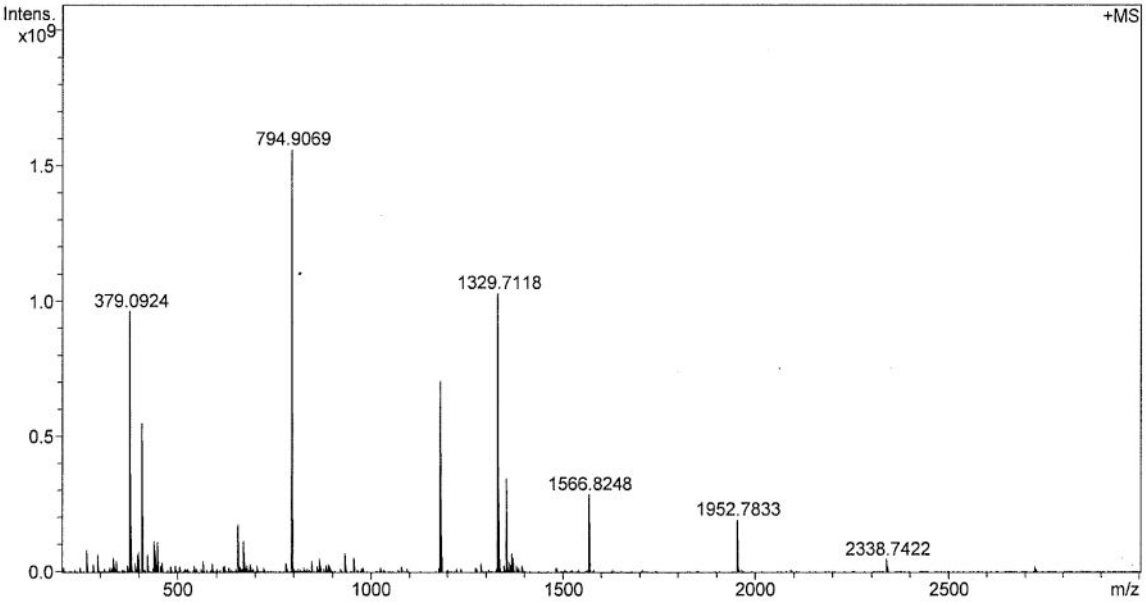


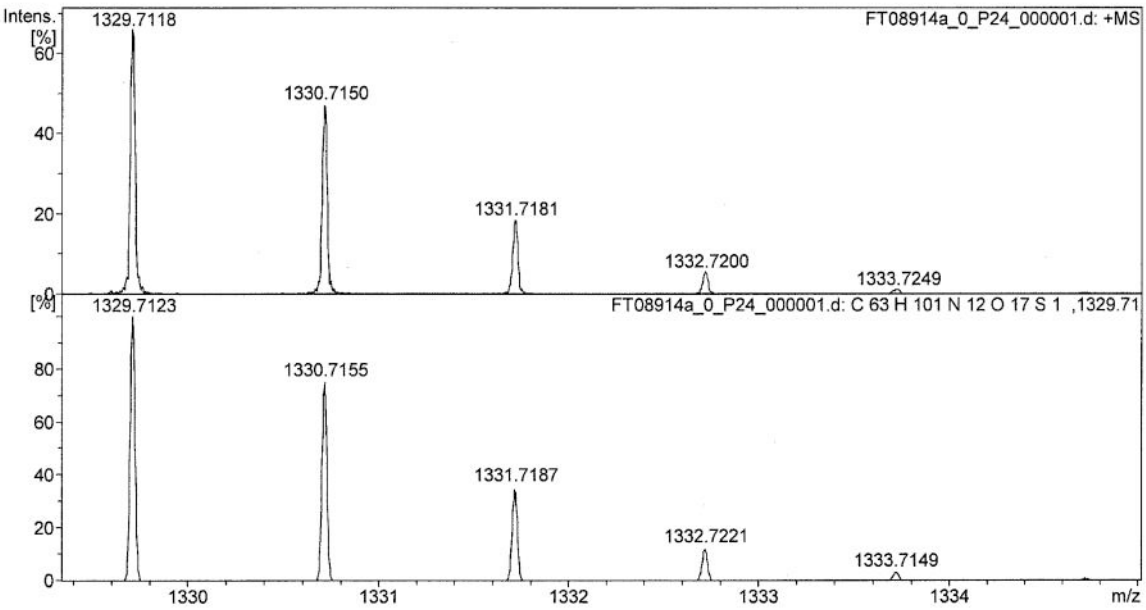


Degradation product S53

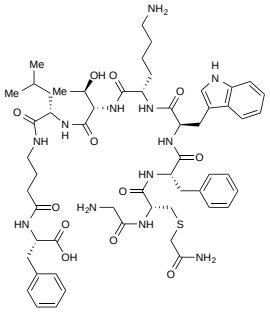


Box 1: Full scale spectra with internal reference peaks (ESI: Na-PFHA 408.9481, 794.9069, 1180.8657, 1952.7834, 2338.7423); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.

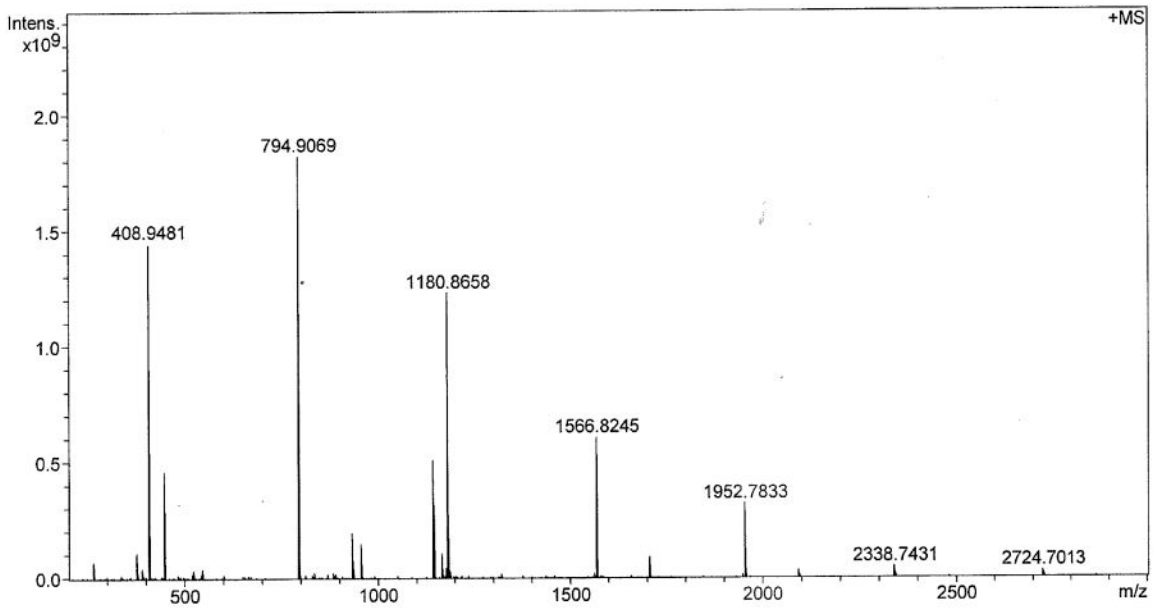


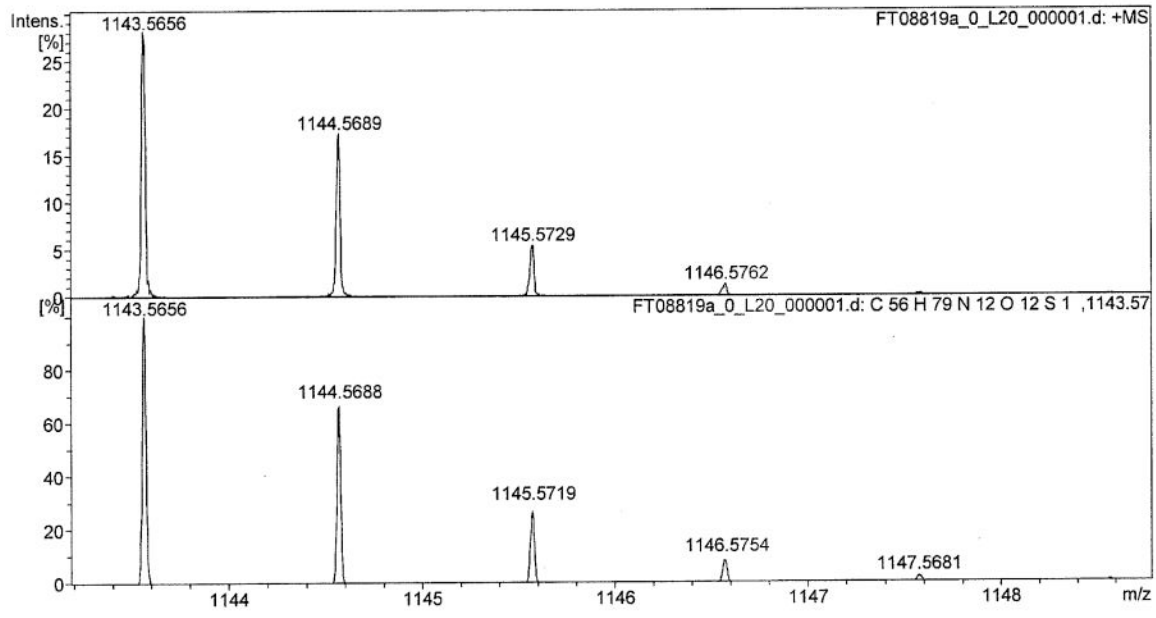


Degradation product S54

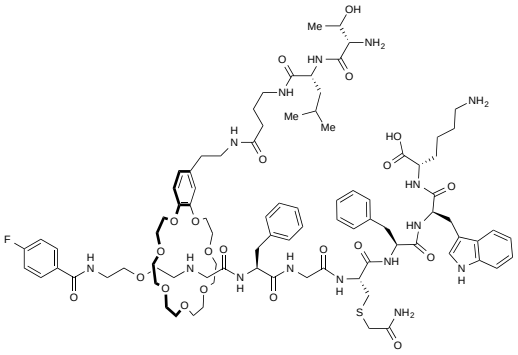


Box 1: Full scale spectra with internal reference peaks (ESI: Na-PFHA 408.9481, 794.9069, 1180.8657, 1566.8246, 1952.7834, 2724.7011); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.

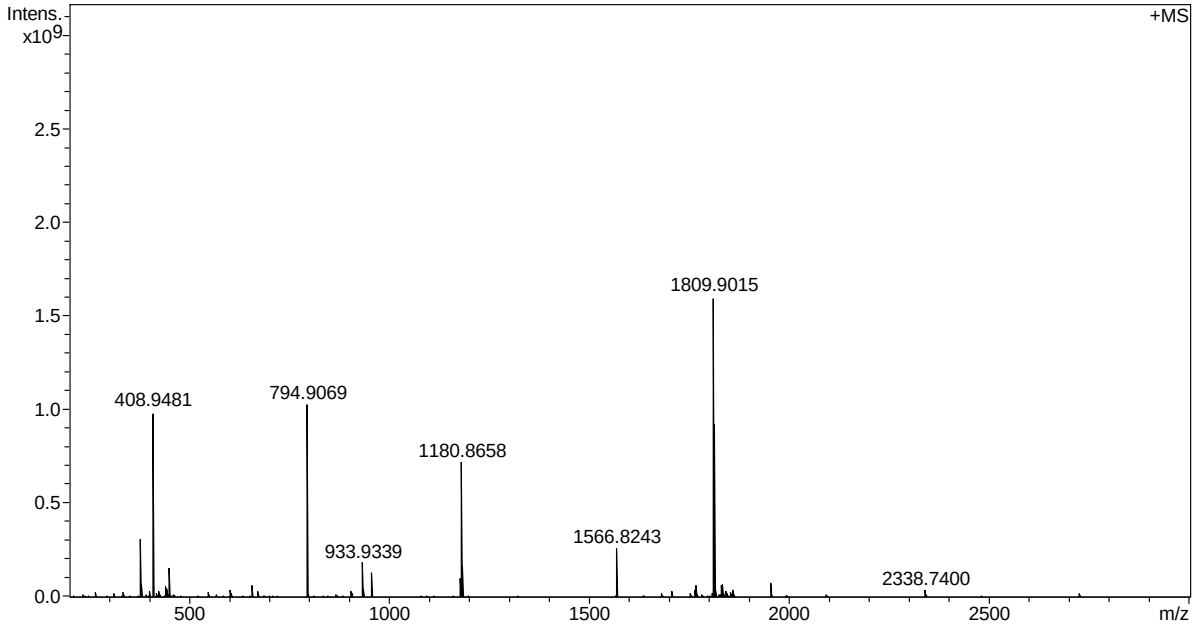


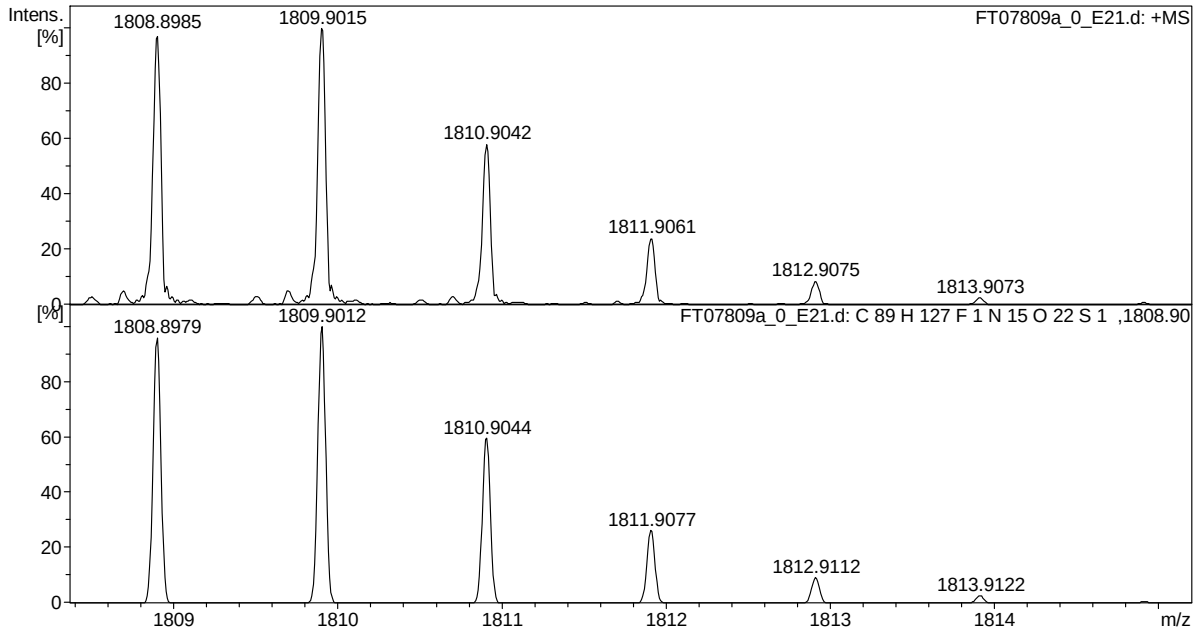


Degradation product S55

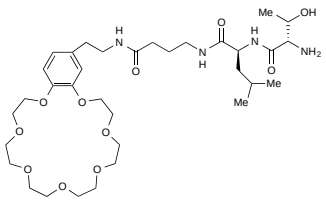


Box 1: Full scale spectra with internal reference peaks (ESI: Na-PFHA 408.9481, 794.9069, 1180.8657, 1566.8246, 1952.7834); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.

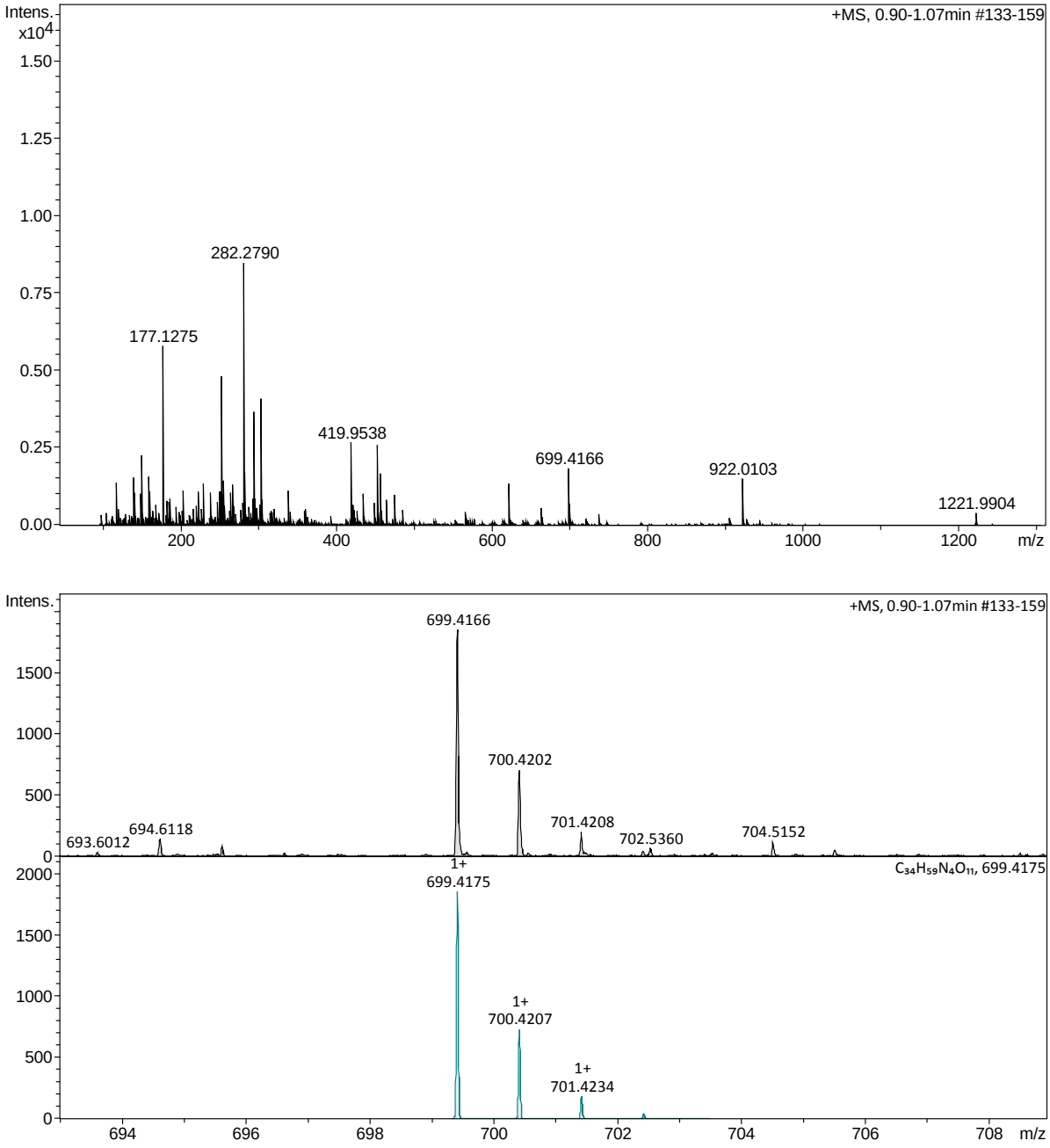




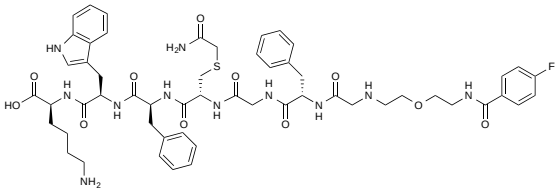
Degradation product S56



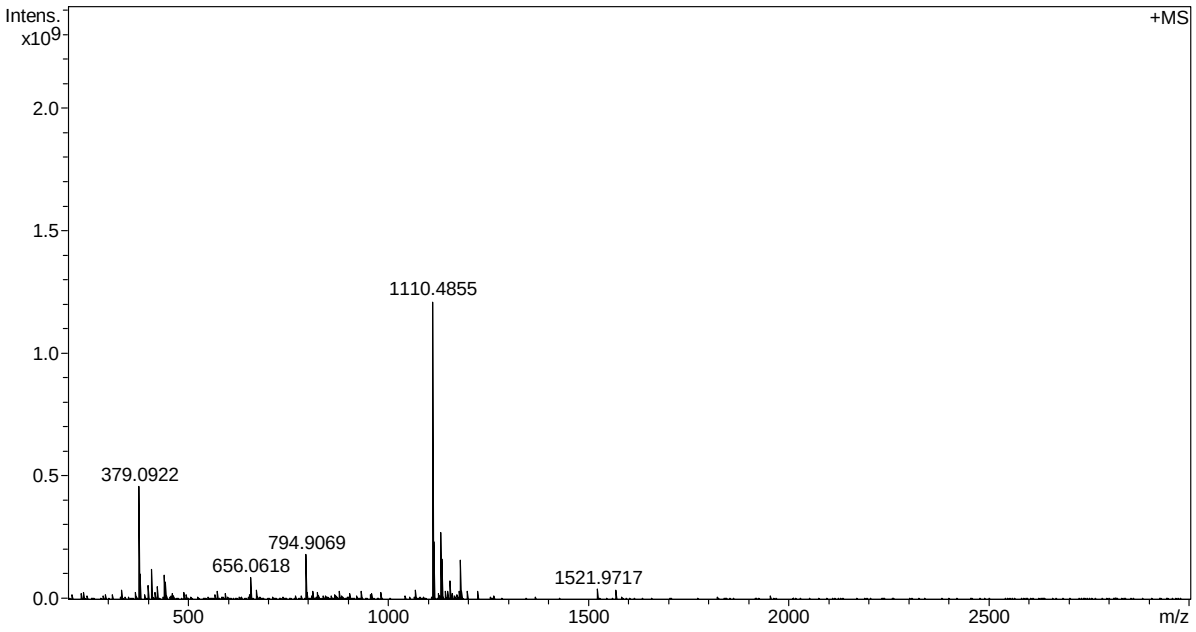
Box 1: Full scale spectra with internal reference peaks (Tunemix (pos) ESI-TOF Spezial 118.0863, 622.0290, 922.0098, 1221.9906); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.

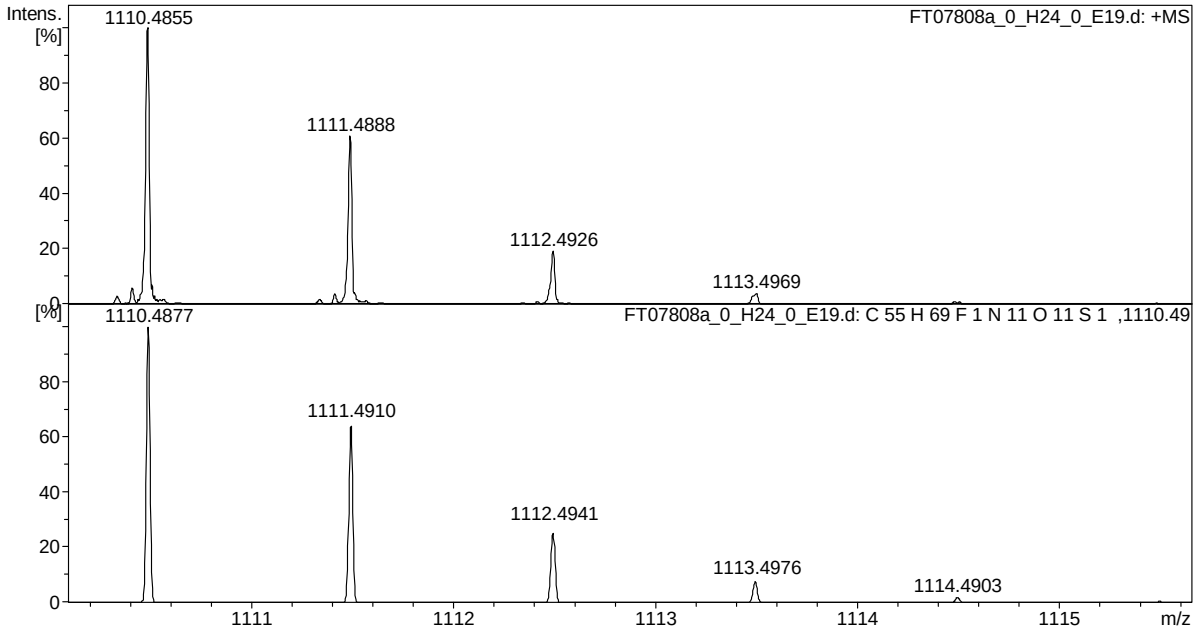


Degradation product S57

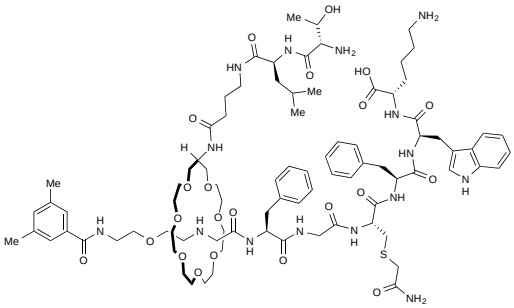


Box 1: Full scale spectra with internal reference peaks (ESI: Na-PFHA 408.9481, 794.9069, 1180.8657, 1566.8246); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.

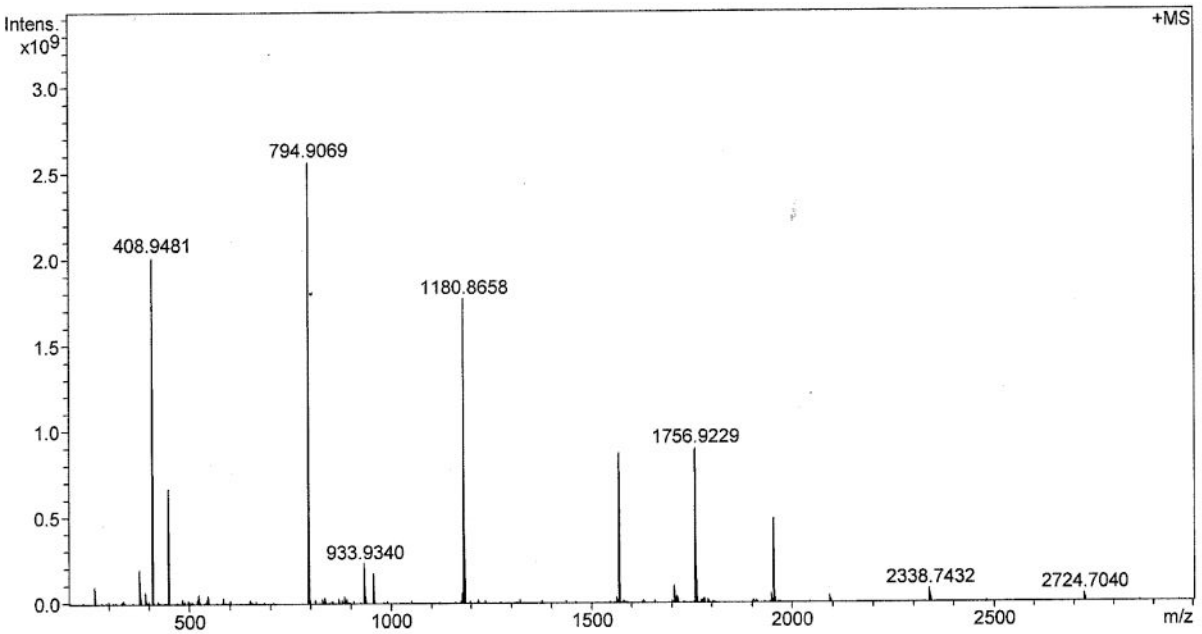


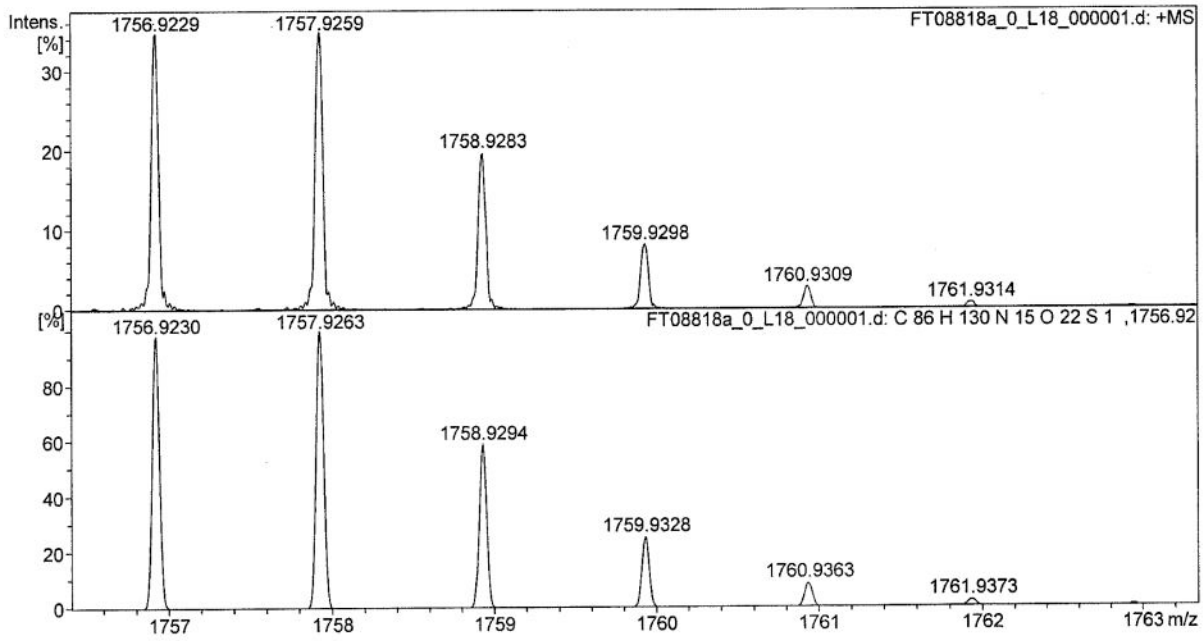


Degradation product S58

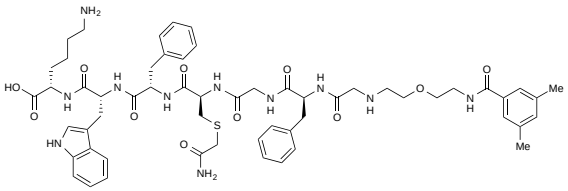


Box 1: Full scale spectra with internal reference peaks (ESI: Na-PFHA 408.9481, 794.9069, 1180.8657, 1566.8246, 1952.7834); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.

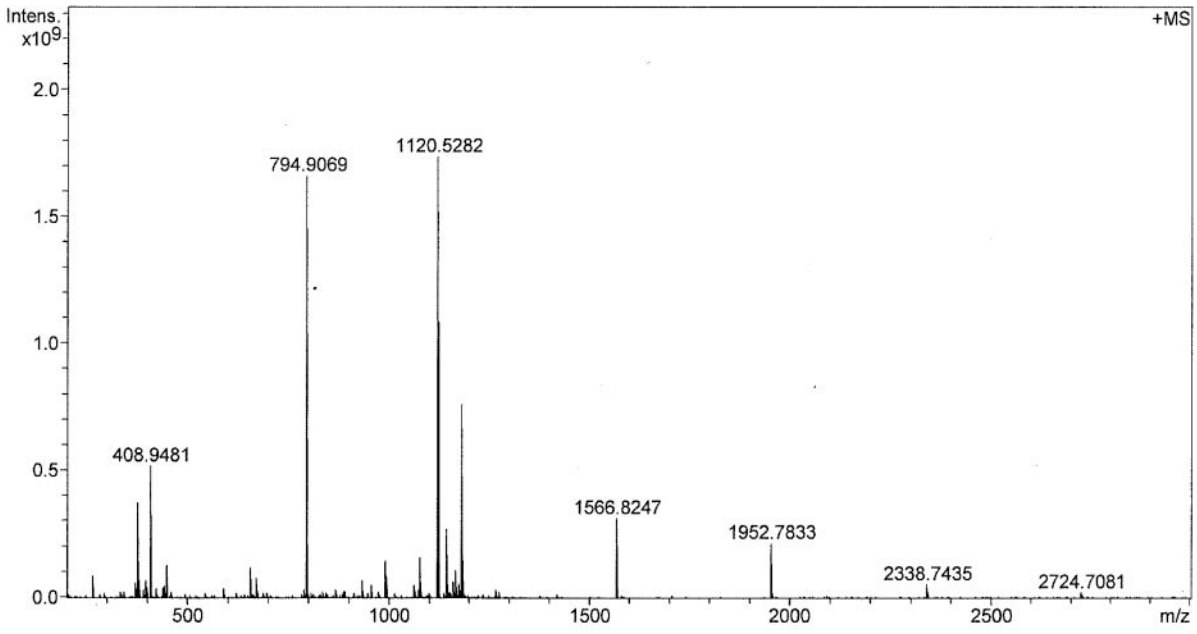


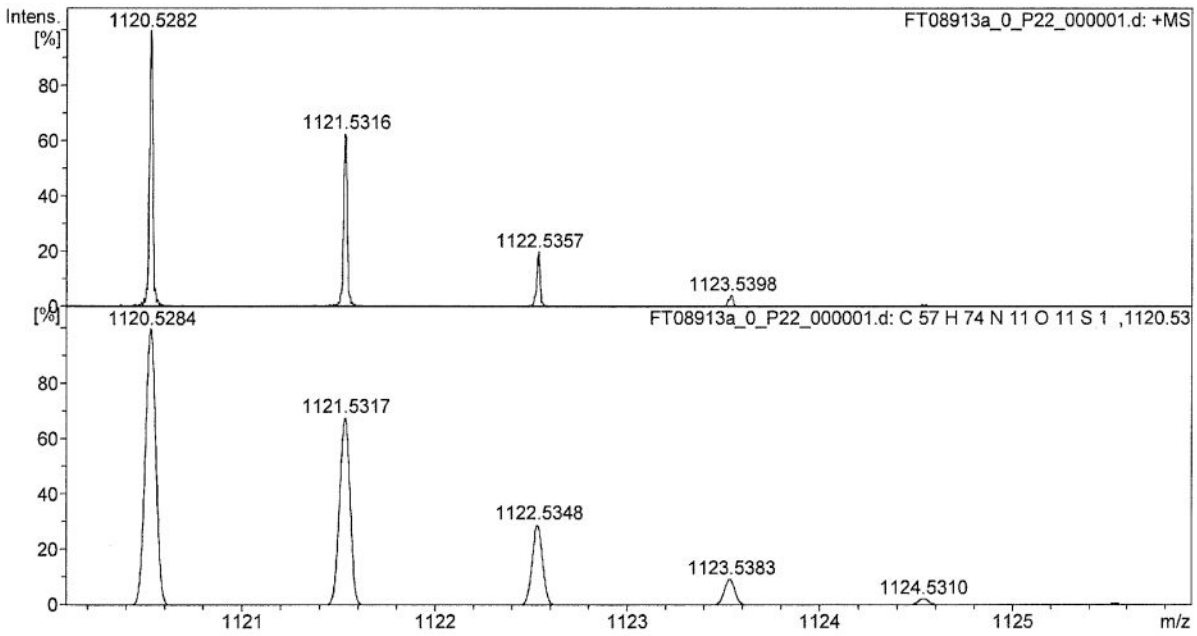


Degradation product S59

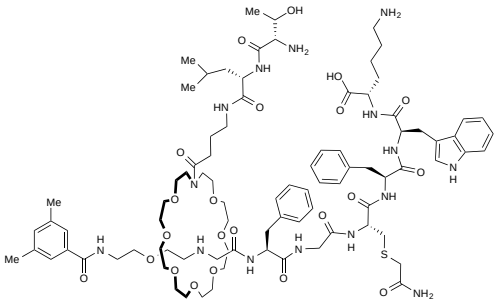


Box 1: Full scale spectra with internal reference peaks (ESI: Na-PFHA 408.9481, 794.9069, 1180.8657, 1566.8246, 1952.7834); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.

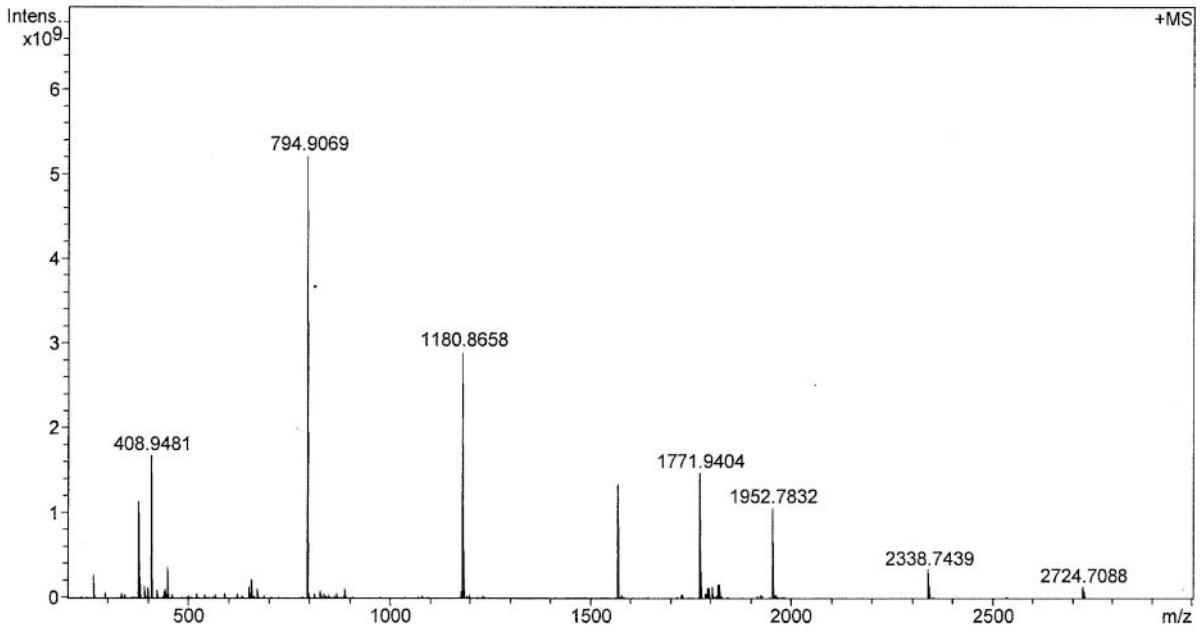


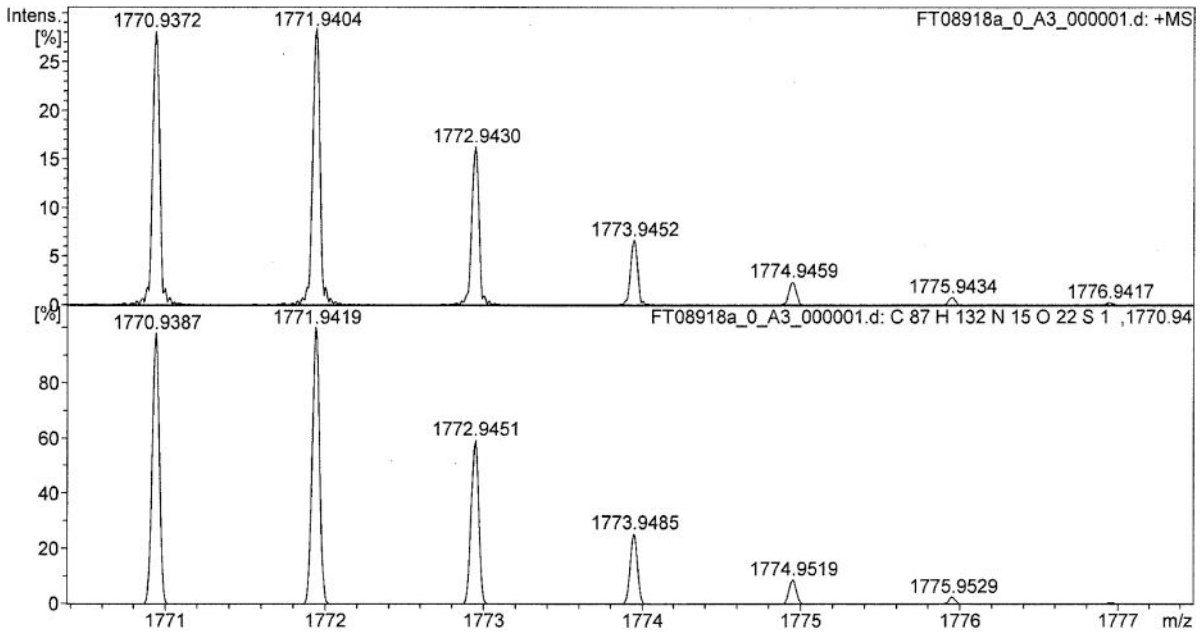


Degradation product S60

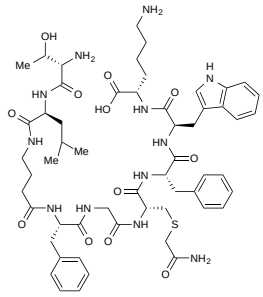


Box 1: Full scale spectra with internal reference peaks (ESI: Na-PFHA 408.9481, 794.9069, 1180.8657, 1566.8246, 1952.7834); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.

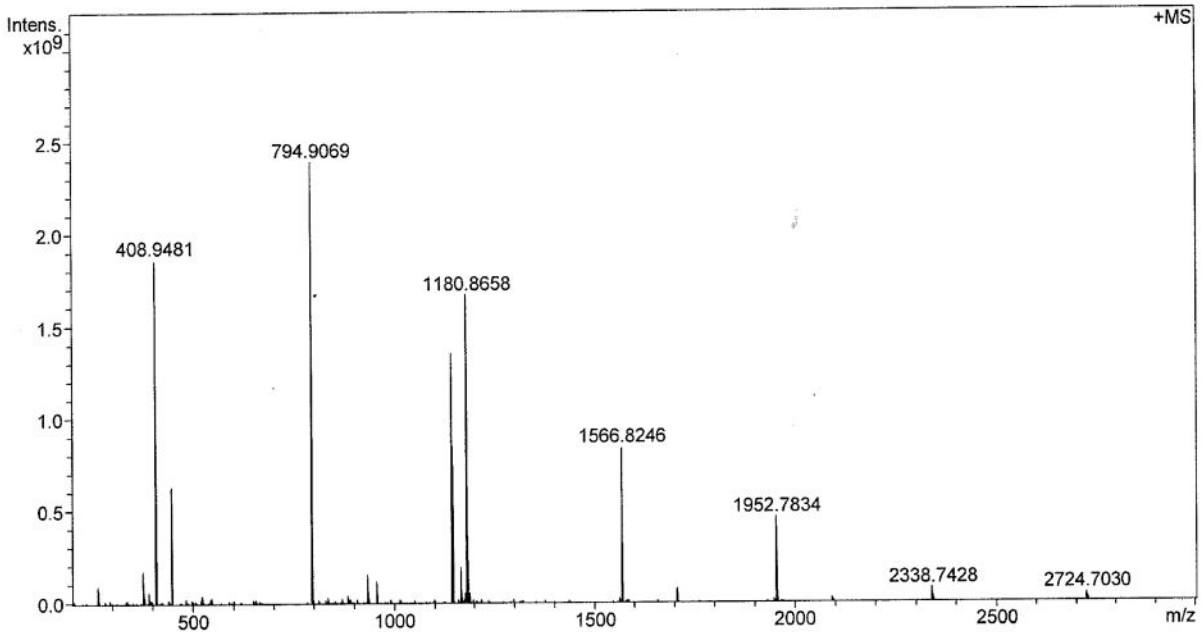


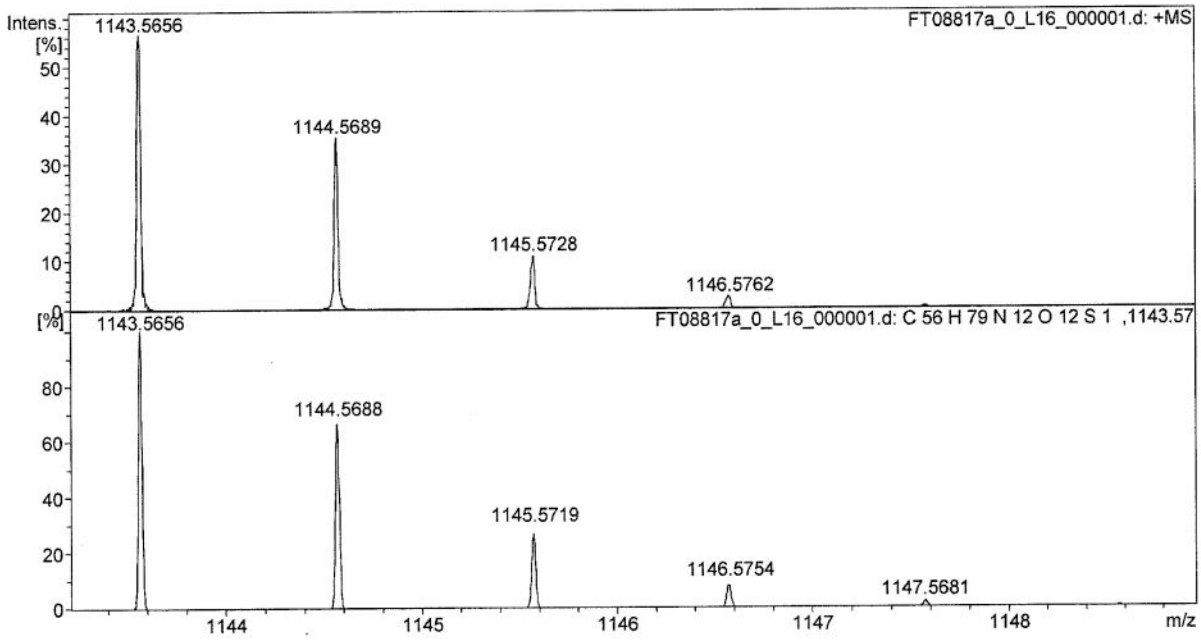


Degradation product S61

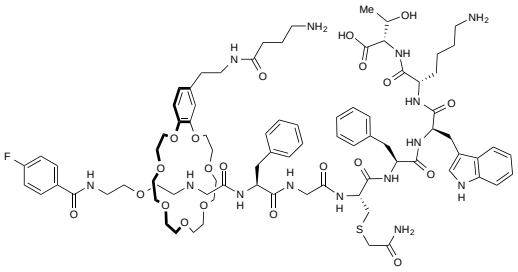


Box 1: Full scale spectra with internal reference peaks (ESI: Na-PFHA 408.9481, 794.9069, 1180.8657, 1566.8246, 1952.7834); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.

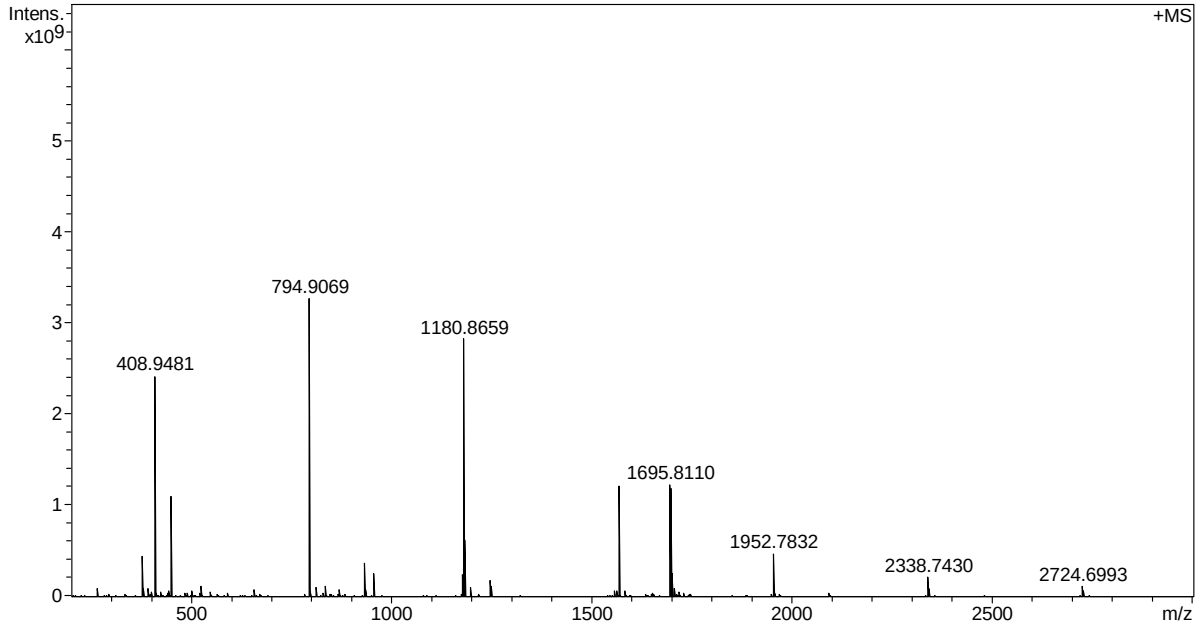


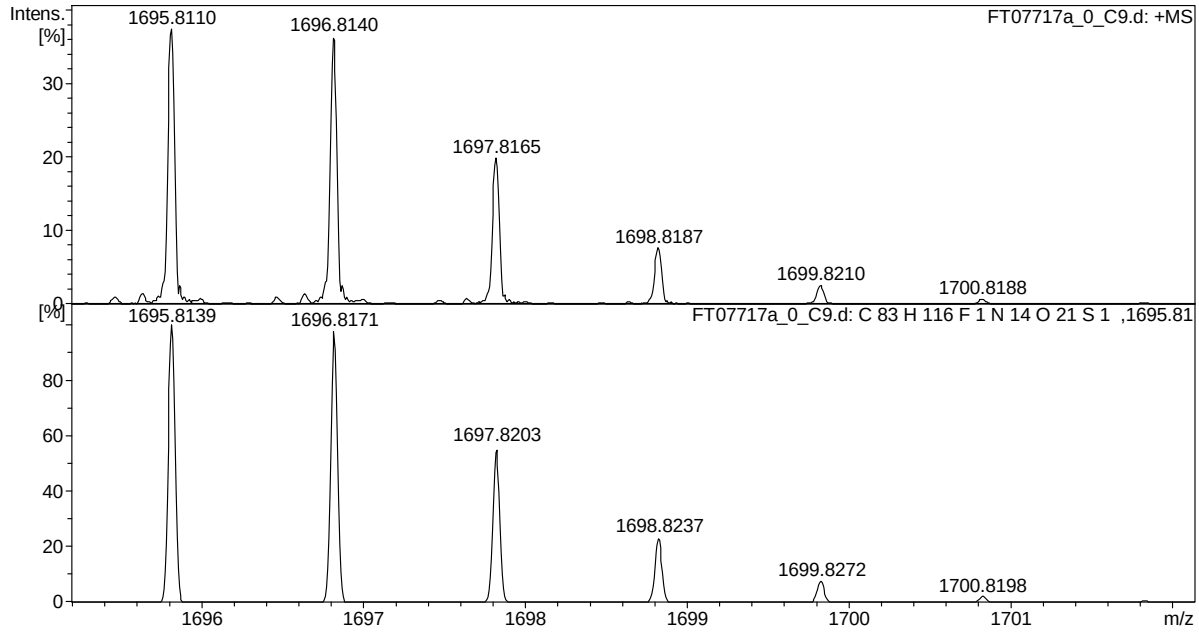


Degradation product S62

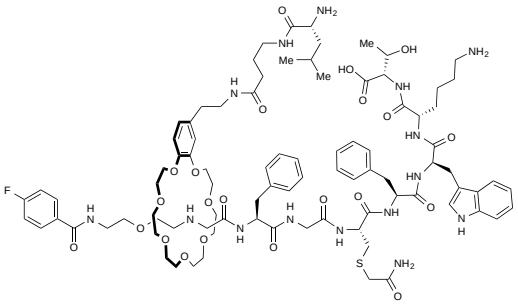


Box 1: Full scale spectra with internal reference peaks (ESI: Na-PFHA 408.9481, 794.9069, 1180.8657, 1566.8246, 1952.7834); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.

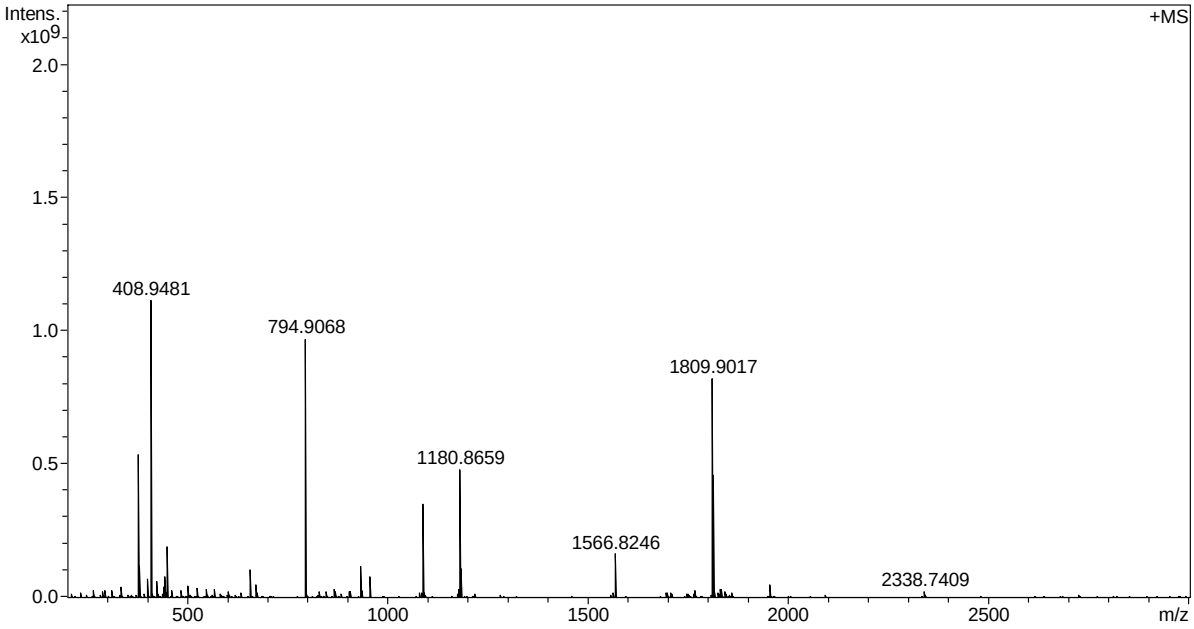


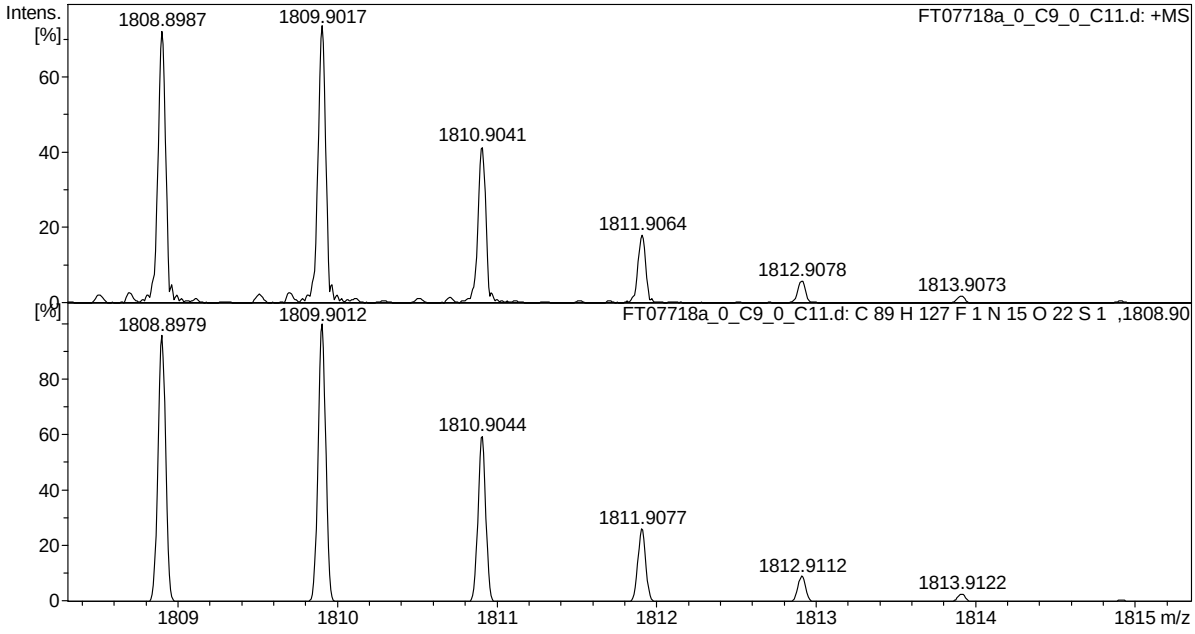


Degradation product S63

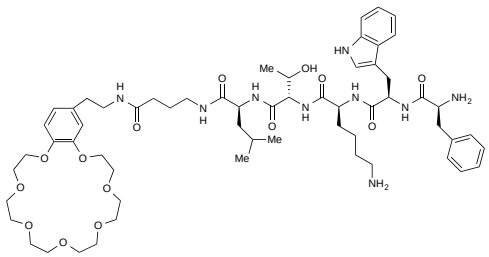


Box 1: Full scale spectra with internal reference peaks (ESI: Na-PFHA 408.9481, 794.9069, 1180.8657, 1566.8246, 1952.7834); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.

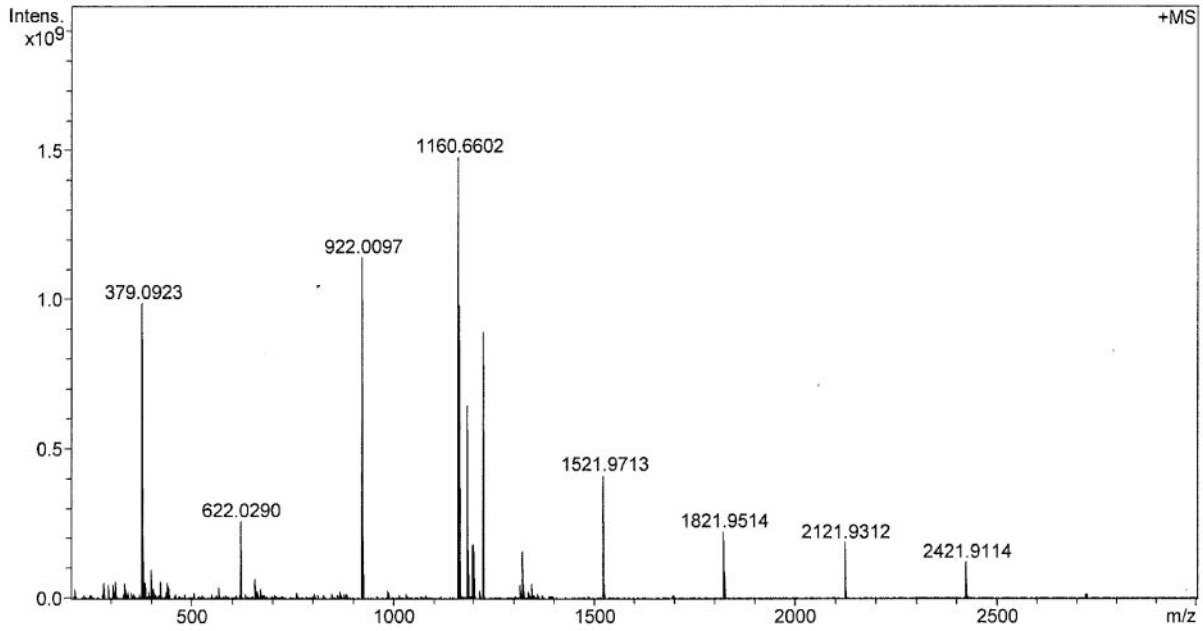


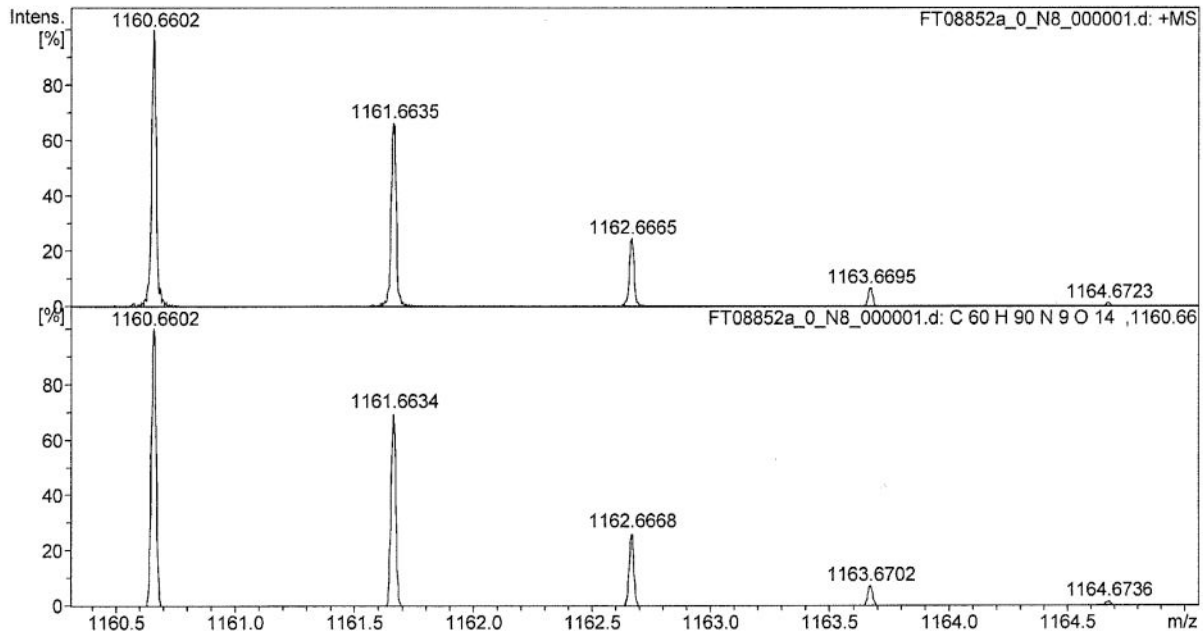


Degradation product S64

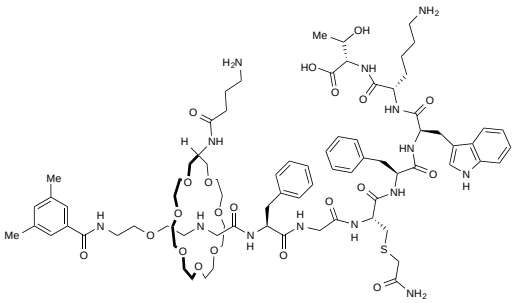


Box 1: Full scale spectra with internal reference peaks (ESI: Tuning Mix ES-TOF (ESI) (pos)DCTB 322.0481, 622.0290, 922.0098, 1221.9906, 1521.9715); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.

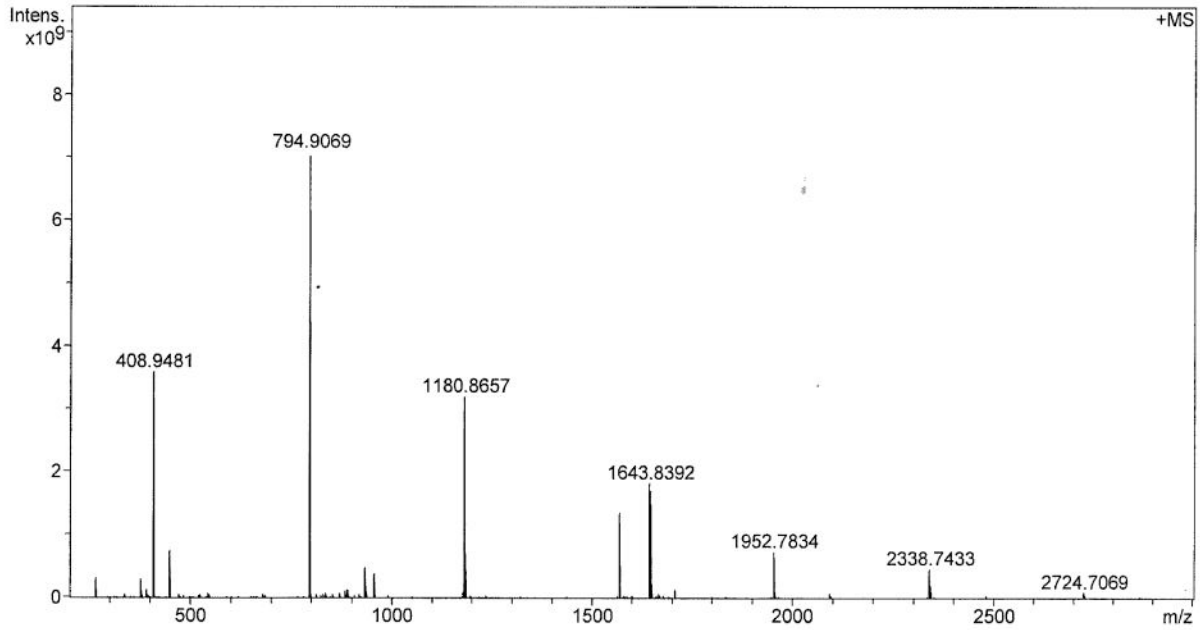


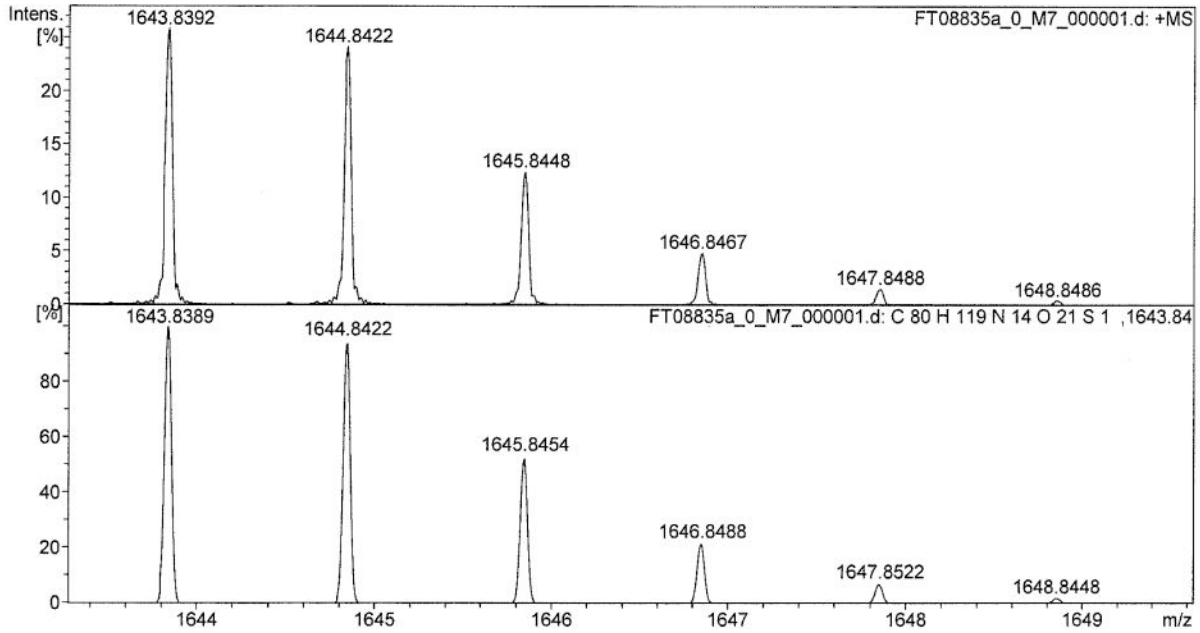


Degradation product S65

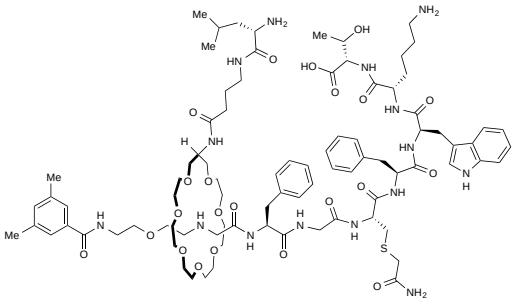


Box 1: Full scale spectra with internal reference peaks (ESI: Na-PFHA 408.9481, 794.9069, 1180.8657, 1566.8246, 1952.7834); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.

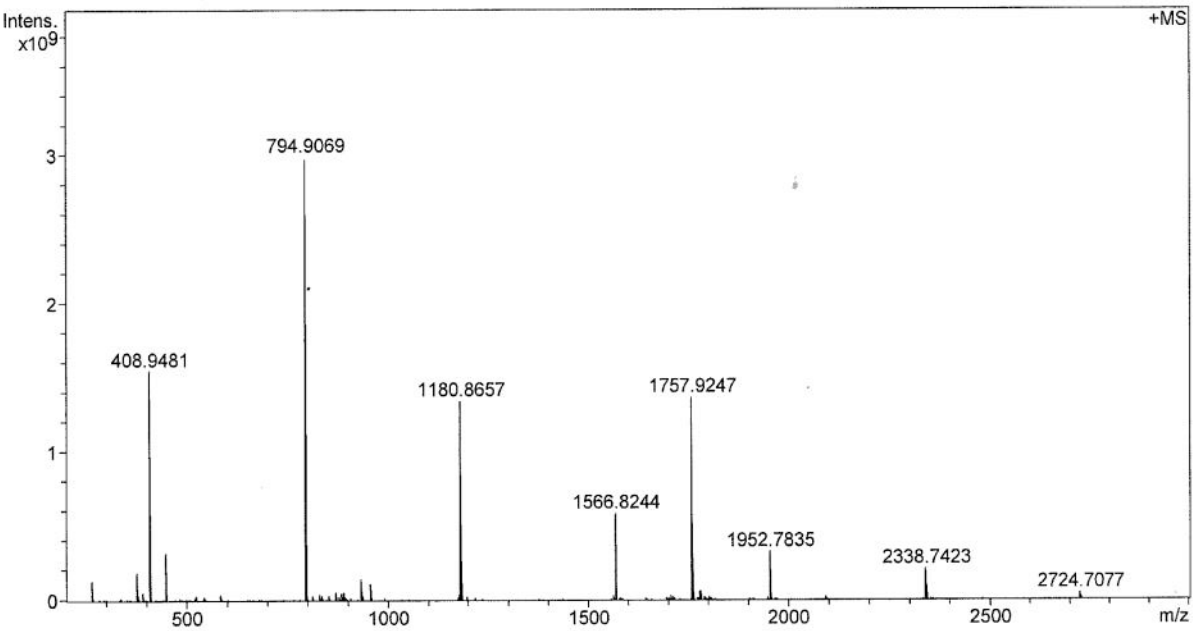


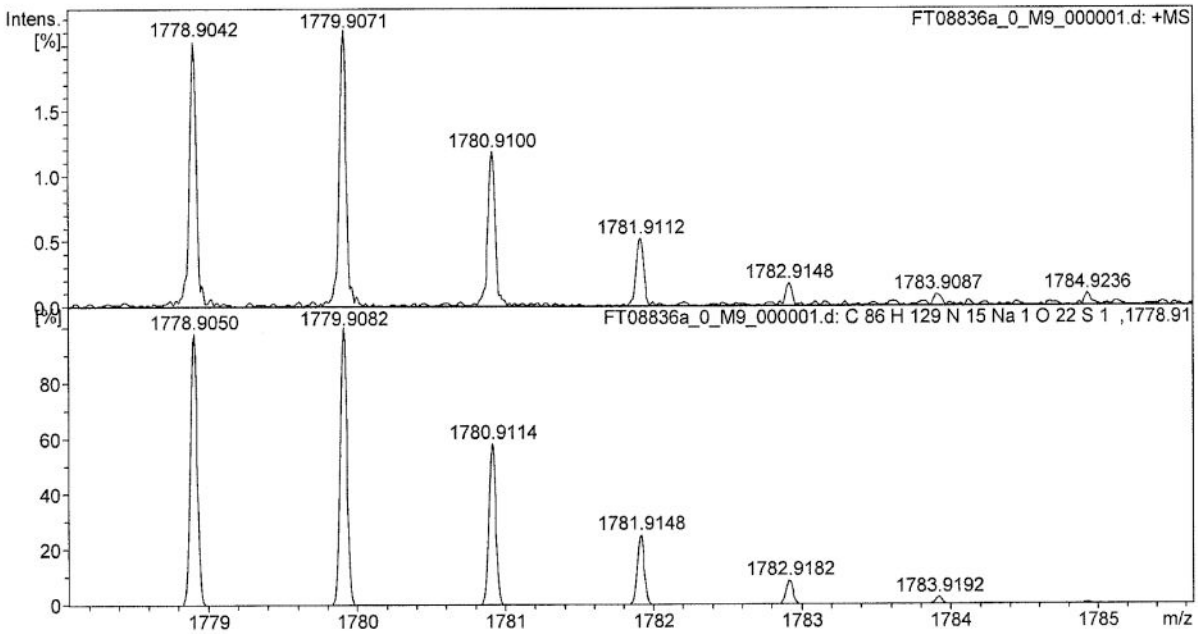


Degradation product S66

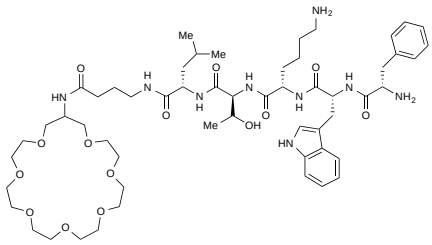


Box 1: Full scale spectra with internal reference peaks (ESI: Na-PFHA 408.9481, 794.9069, 1180.8657, 1952.7834, 2338.7423§); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.

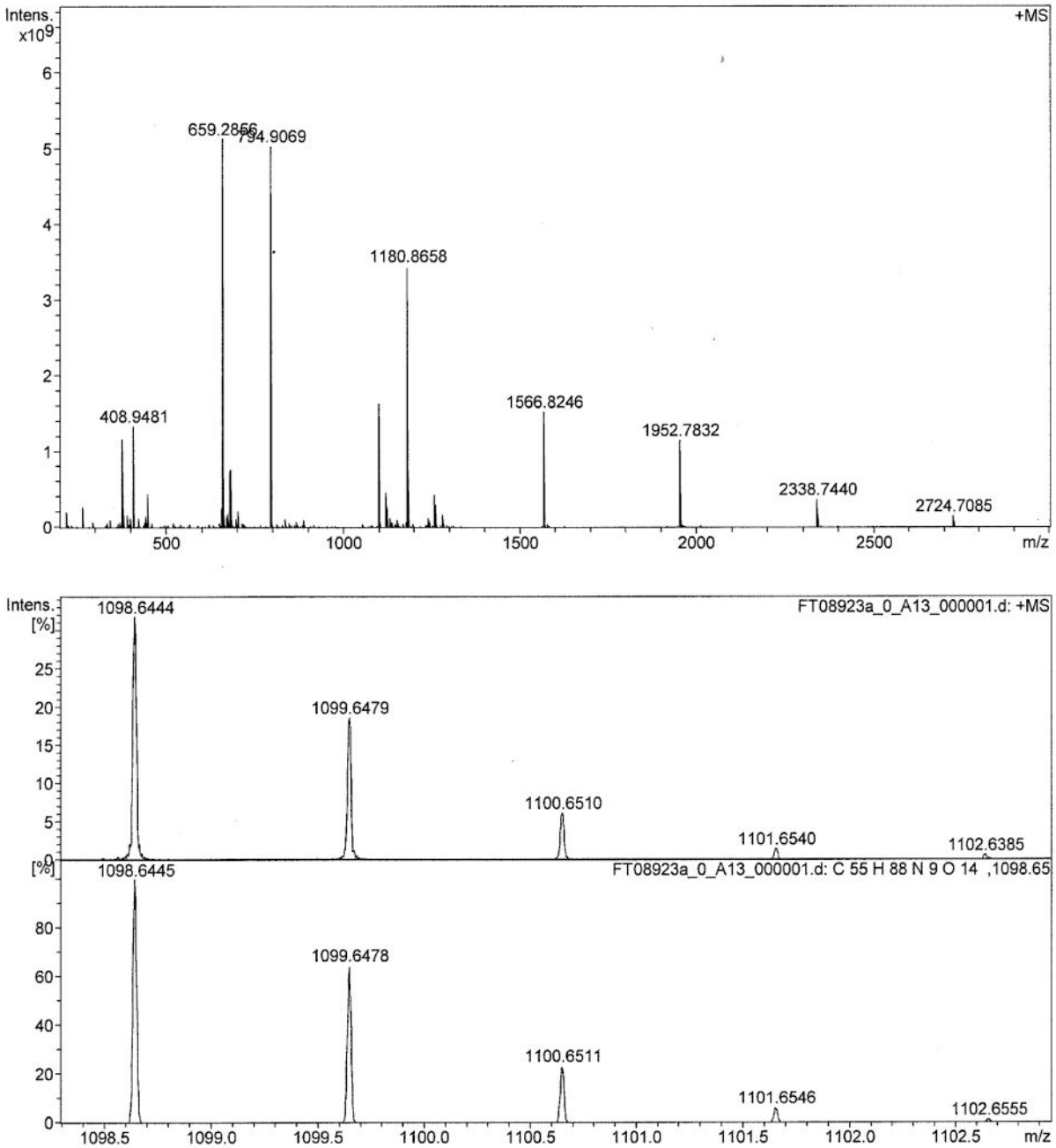




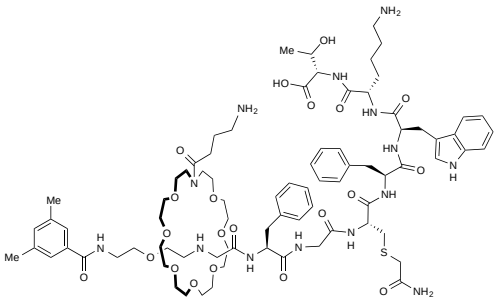
Degradation product S67



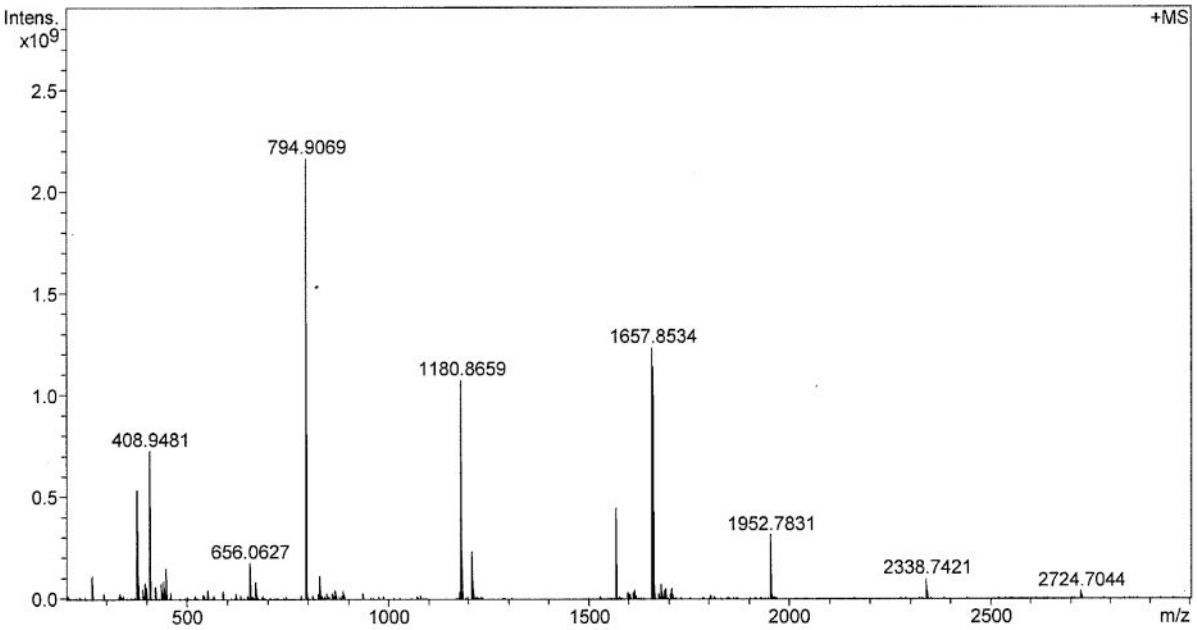
Box 1: Full scale spectra with internal reference peaks (ESI: Na-PFHA 408.9481, 794.9069, 1180.8657, 1566.8246, 1952.7834); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.

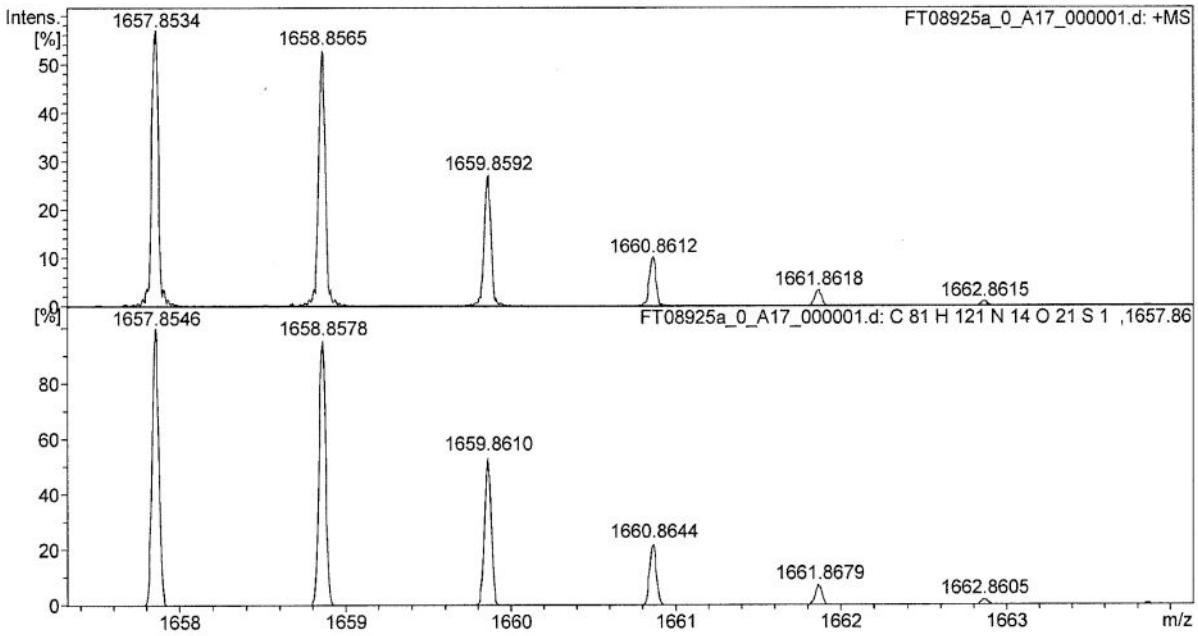


Degradation product S68

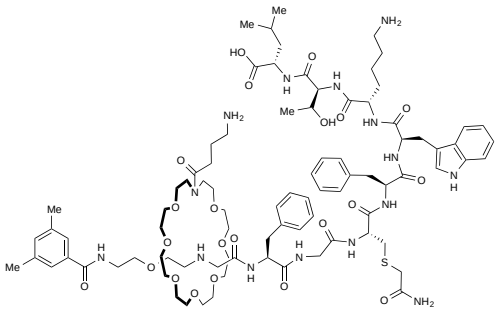


Box 1: Full scale spectra with internal reference peaks (ESI: Na-PFHA 408.9481, 794.9069, 1180.8657, 1566.8246, 2338.7423); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.

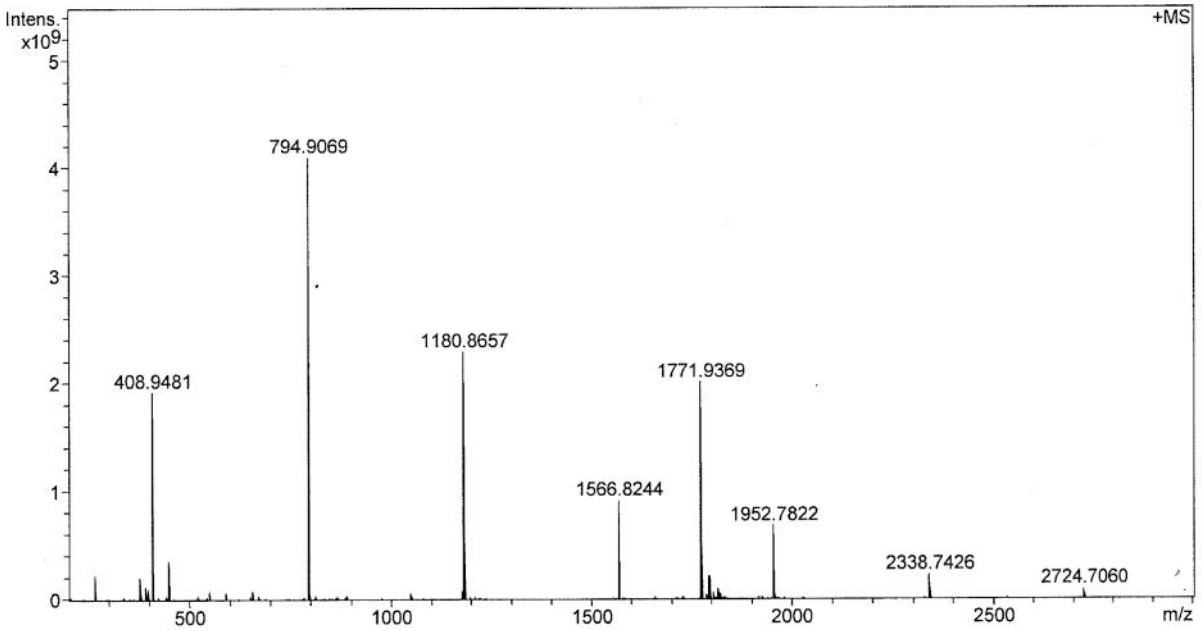


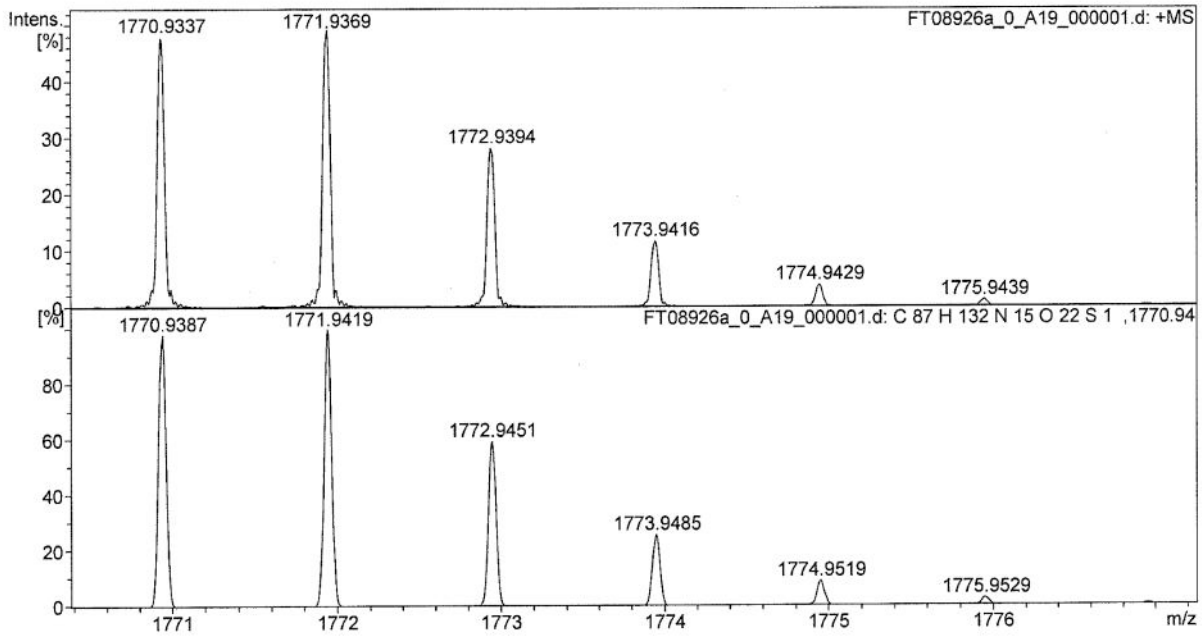


Degradation product S69

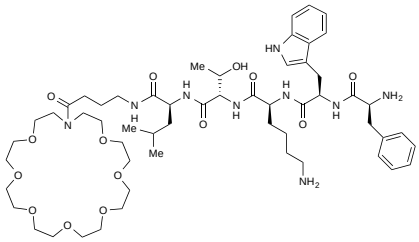


Box 1: Full scale spectra with internal reference peaks (ESI: Na-PFHA 408.9481, 794.9069, 1180.8657, 1566.8246, 2338.7423); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.

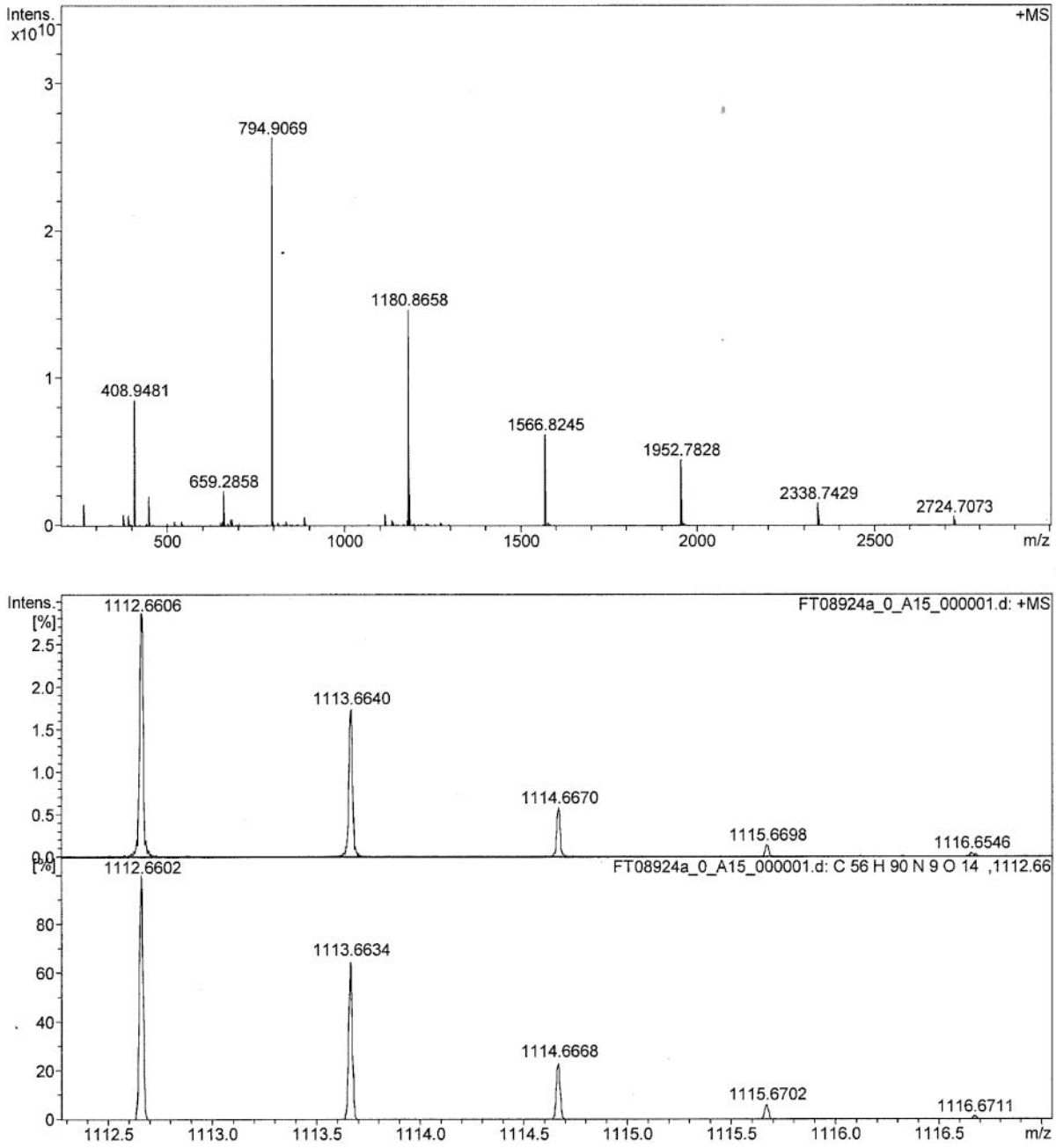




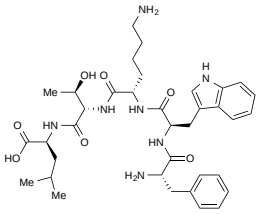
Degradation product S70



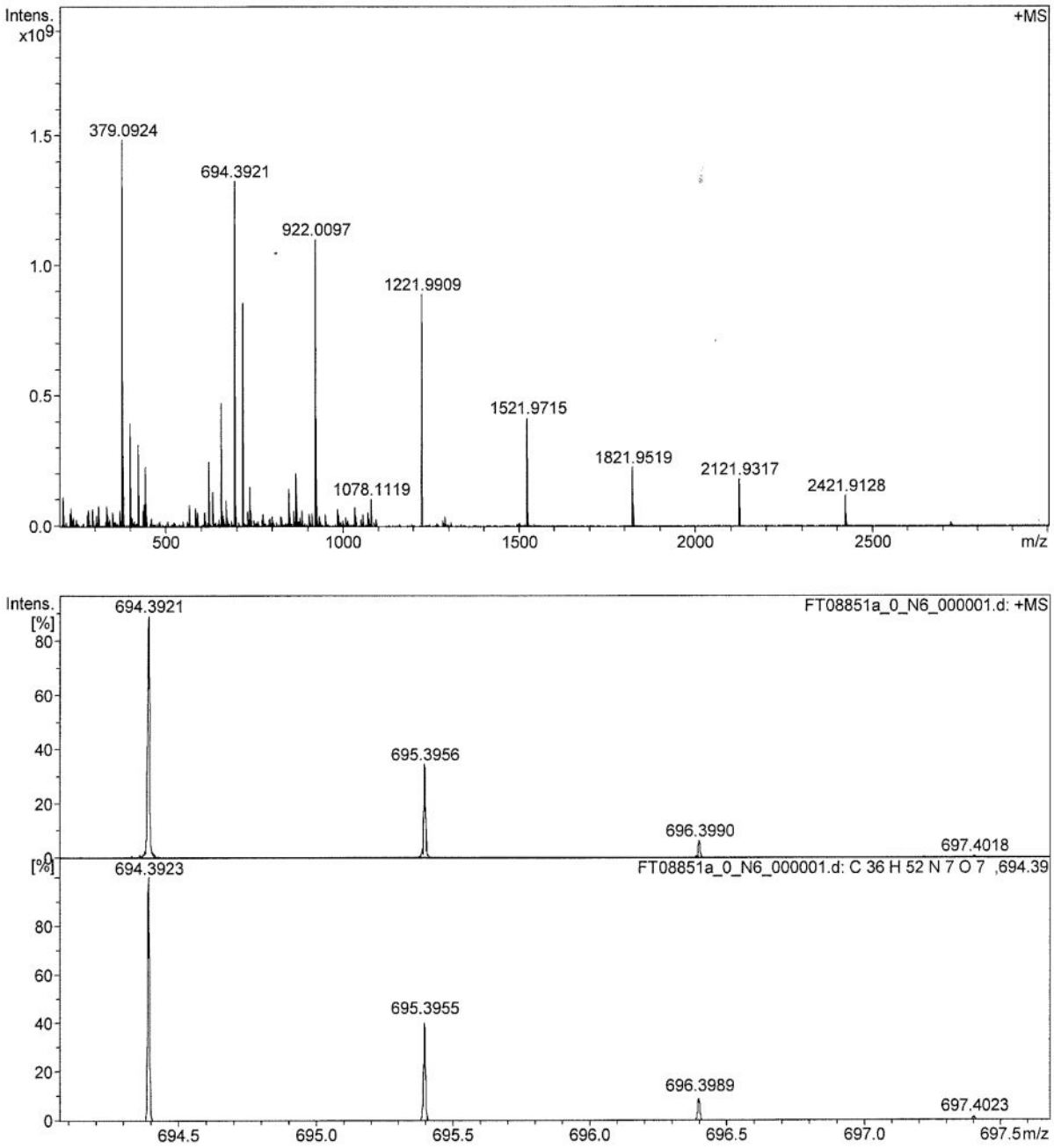
Box 1: Full scale spectra with internal reference peaks (ESI: Na-PFHA 408.9481, 794.9069, 1180.8657, 1566.8246); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.



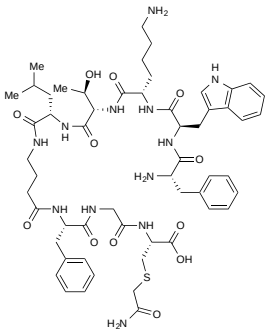
Degradation product S71



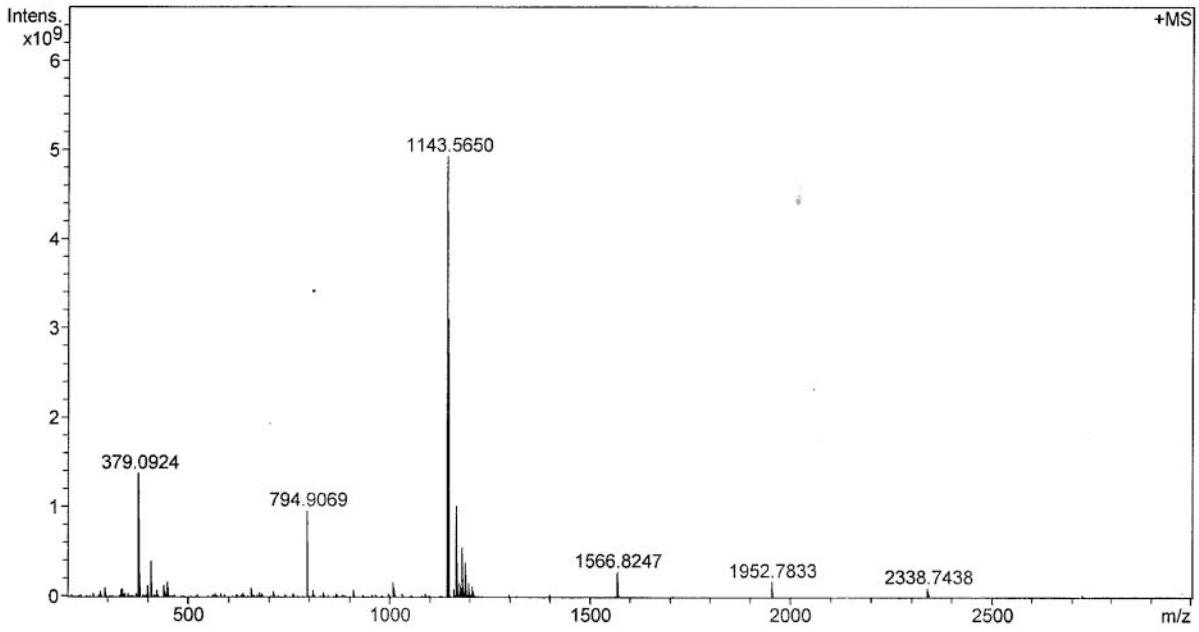
Box 1: Full scale spectra with internal reference peaks (ESI: Tuning Mix ES-TOF (ESI) (pos)DCTB 322.0481, 622.0290, 922.0098, 1521.9715); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.

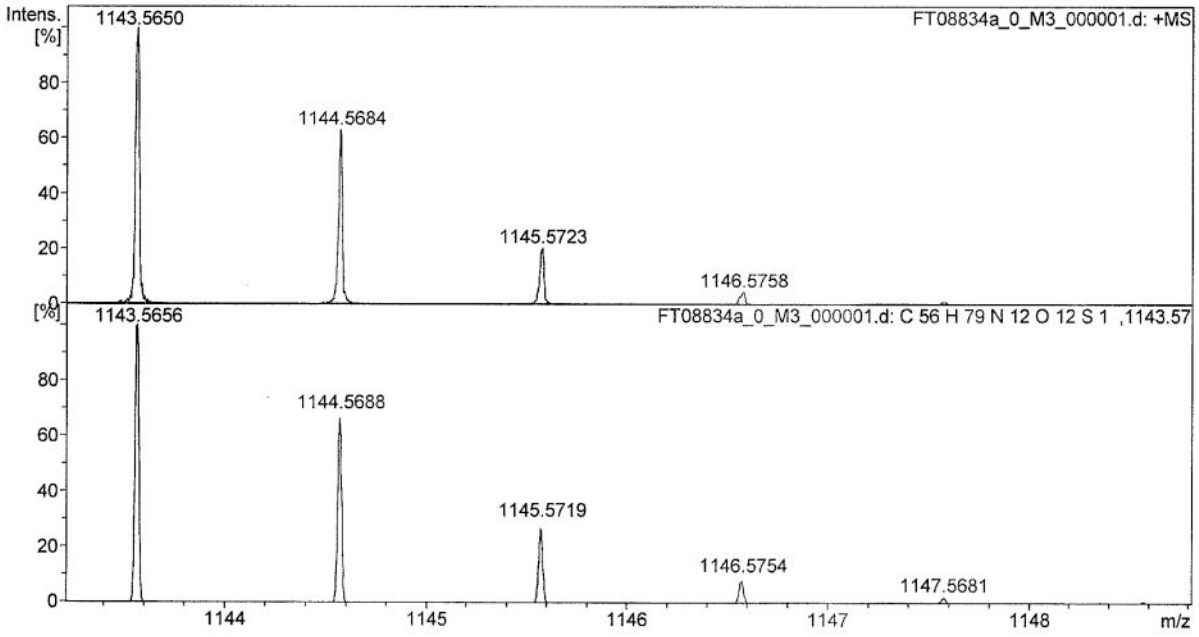


Degradation product S72

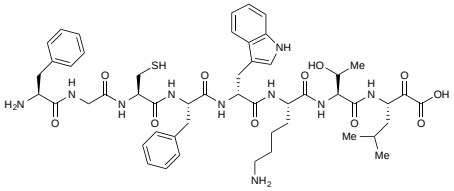


Box 1: Full scale spectra with internal reference peaks (ESI: Na-PFHA 408.9481, 794.9069, 1180.8657, 1566.8246, 1952.7834); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.

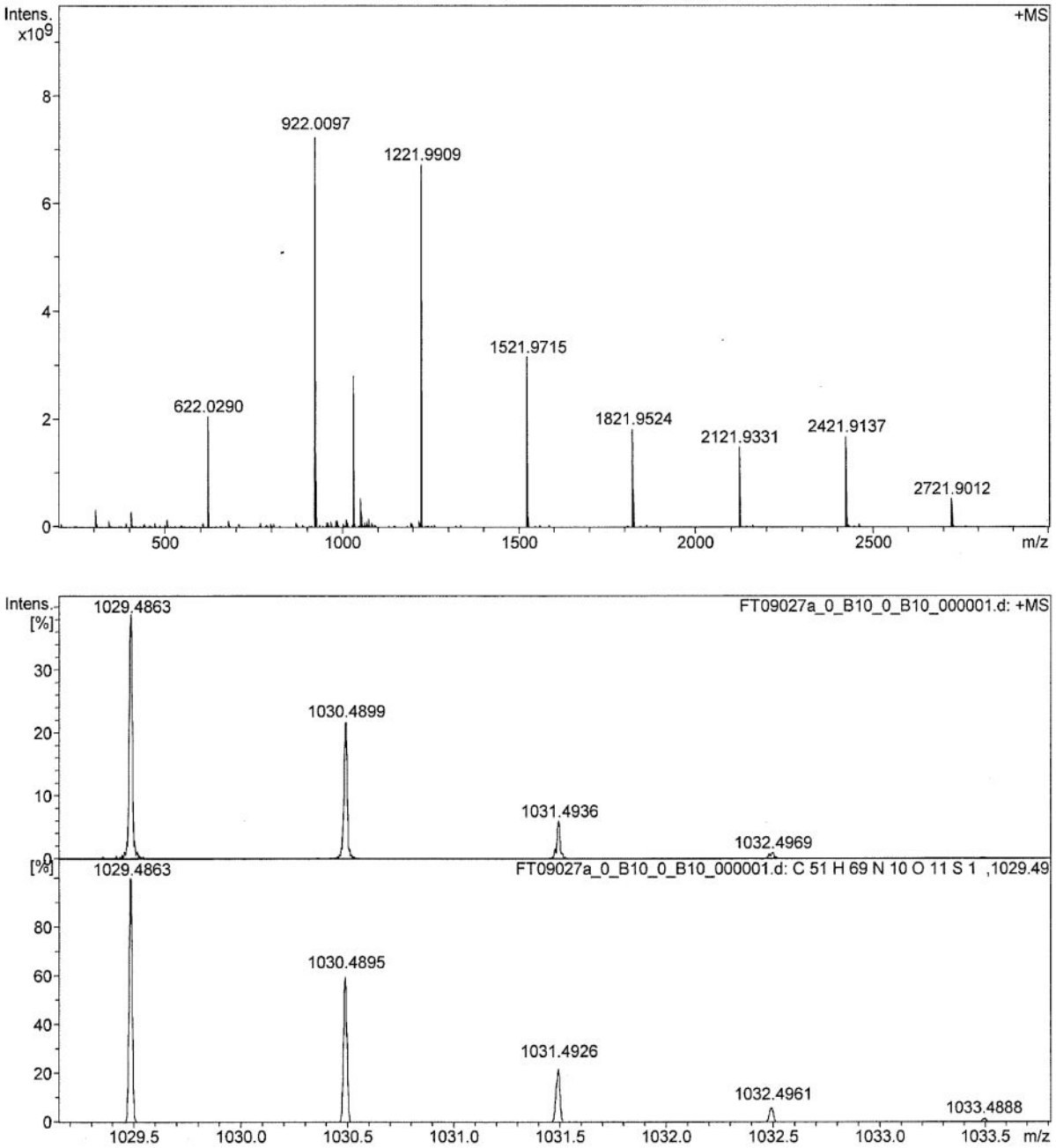




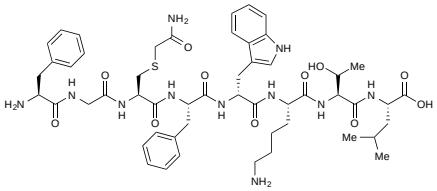
Linear peptide α-ketocacid S73



Box 1: Full scale spectra with internal reference peaks (ESI: Tuning Mix ES-TOF (ESI) (pos)DCTB 622.0290, 922.0098, 1221.9906, 1521.9715, 1821.9523, 2121.9332, 2421.9140); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.



Linear peptide R1



Box 1: Full scale spectra with internal reference peaks (ESI: Na-PFHA 408.9481, 794.9069, 1180.8657, 1566.8246, 1952.7834, 2338.7423); **Box 2:** Measured isotopic pattern of the product peak; **Box 3:** Calculated isotopic pattern of the product peak.